ZMLOX4 AND *ZMLOX5* GENES PLAY CONTRASTING ROLES IN DEFENSE AGAINST *COLLETOTRICHUM GRAMINICOLA*

A Thesis

by

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Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

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May 2018

Major Subject: Plant Pathology

ABSTRACT

Plant lipoxygenases (LOX) catalyze the production of oxylipins through the oxidization of polyunsaturated fatty acids (PUFA) that are important in plant development and defense. LOXs can be divided into two groups, depending on which carbon in the PUFA carbon chain the enzyme oxidizes. Within the two groups, 13-LOX, which oxidizes the 13th carbon in the chain, is better known than 9-LOX, which oxidizes the 9th carbon. Two maize LOX gene ohnologues, *ZmLOX4* and *ZmLOX5*, are members of the 9-LOX subfamily and share over 94% identity at the amino acid level. Remarkably, despite this extreme homology, these genes have contrasting roles during defense against the causal agent of anthracnose leaf and stalk blight, *Colletotrichum graminicola*. Functional analyses of knock-out mutants revealed that *ZmLOX4* is required for defense against this pathogen, while *ZmLOX5* facilitates fungal pathogenicity. The major objectives of this study were: (1) to identify the oxylipin signature produced by the *ZmLOX4* and *ZmLOX5* pathways and (2) to characterize the downstream molecular and biochemical processes that underlie their contrasting functions against *C. graminicola*. Among the eleven 9-oxylipins detected during leaf infections, none were found to be specifically produced by the *ZmLOX4* or *ZmLOX5* pathways, suggesting that as-yet-uncharacterized 9-oxylipins are likely the products of these two homologs. Interestingly, disruption of *ZmLOX4* results in lower accumulation of the major defense hormones, salicylic acid (SA) and jasmonic acid-isoleucine (JA-Ile), while mutation of *ZmLOX5* results in increased accumulation of SA and JA-Ile. These results suggest that yet-unknown ZmLOX5-produced oxylipins negatively regulate synthesis of both SA and JA, and ZmLOX4-derived oxylipins are positive regulators of SA-dependent defense against *C. graminicola*. Supporting the hypothesis that *lox4* mutants are more susceptible due to lower than normal levels of SA,

exogenous SA treatment of *lox4* mutant partially rescued the mutant susceptibility phenotype. RNA-sequencing and shot-gun proteomic analysis of infected leaves revealed transcripts and proteins that are differentially expressed in *lox4* and *lox5* mutants compared to wild-type. The results indicated that in addition to aberrant SA and JA synthesis, the genes and proteins related to production and scavenging of reactive oxygen species (ROS) were also among the most altered outcomes of the mutations. In agreement with these results, *lox5* mutant plants produced greater levels of hydrogen peroxide in response to *C. graminicola,* while *lox4* mutants were less responsive in terms of ROS accumulation. Additionally, it was found that conidia germinate at a faster rate on both *lox4* and *lox5* mutant plants, suggesting that these two genes regulate early stages of infection processes. In conclusion, the data suggest that these two nearly-identical 9-LOXs produce some as-yet unknown oxylipin signals that differentially regulate maize defense response to *C. graminicola* most notably via regulation of ROS and SA.

DEDICATION

This is dedicated to my father, Ipung Punto Yuwono, who passed away during the course of this study and to my mother, Martiana Nefiyanti.

ACKNOWLEDGEMENTS

I would like to thank my Graduate Committee chair, Dr. Mike Kolomiets and my committee members, Dr. Charles Kenerley, Dr. Daniel Ebbole, and Dr. Ping He for their counsel and support throughout the course of this research. Thank you as well to my friends and colleagues in the Department of Plant Pathology along with the faculty and staff.

I would like to thank all my undergraduate assistants I've had throughout the course of my study, Jonathan Hilton, Kirsten Higgs, Kevin Wei, Jose Torres, Avery Willard, and Franko Jonker, for without their help I would not be able to complete this study.

I would like to thank my friends Amanda Gowell and Jack Wilson for their contributions and help in proofreading this document.

Lastly, but no less important, I would like to thank the goat herding Sufi Kaldi for discovering coffee beans. I, and likely millions of others, would not have been able to do anything without it.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a committee consisting of Professor Michael Kolomiets, who acted as chair, and its members, Professors Charles Kenerley and Daniel Ebbole of the Department of Plant Pathology and Microbiology, and Professor Ping He of the Department of Biochemistry and Biophysics.

Transcript sequencing was conducted by the Texas A&M Agrilife Research Genomics and Bioinformatics Services.

Extraction of proteins for proteome analysis was conducted by Dr. Yong-Soon Park.

LC/MS-MS work for proteomics was conducted by the laboratory of Professor Joshua Yuan.

LC/MS-MS work for hormones, cis-acting element information, and genewise negative binomial generalized linear modeling was conducted by Dr. Eli Borrego.

All other work was conducted independently by the student.

Graduate study was supported by a fellowship from Texas A&M University.

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1. INTRODUCTION

Maize (*Zea mays* subspecies *mays*) is an economically significant grain plant originally domesticated in Mesoamerica, but now cultivated across the world. Maize is valuable as a staple crop (Kimanya et al., 2008), feed (Placinta et al., 1999), and for generating ethanol biofuel (Badger, 2002). Maize production is threatened by climate change (Msowoya et al., 2016), insects (Brown and Gange, 1990), and plant pathogens (Tsai and Falk, 1998). Improvement of maize productivity will require detailed knowledge about the mechanisms of defense responses against both biotic and abiotic threats. This will involve the identification of novel gene targets and metabolite pathways that may be exploited to increase yield while protecting the plant against diverse stressors. A major threat world-wide to maize is the plant pathogenic fungus, *Colletotrichum graminicola*. *C. graminicola* is an ascomycete that has been found to be extremely devastating under Brazilian environmental conditions (Cota et al., 2012). Additionally, this pathogen has caused an estimated yield loss of around 47.9 million bushels in the top 21 US corn-producing states and Ontario, Canada in 2013 (Mueller and Wise, 2014). This pathogen is a hemibiotrophic pathogen, initiating infection in a biotrophic phase before transitioning into a necrotrophic phase as disease progresses. This fungus causes two major diseases in maize, anthracnose leaf blight (ALB) and anthracnose stalk rot (ASR). ALB is a disease that is characterized by the appearance of extensive necrotic lesions on the leaves, reducing the photosynthetic area of the plant. ASR reduces yield primarily due stalk lodging and allows the pathogen to consume reserves in the stalk that would be otherwise used to fill the grain (Cota et al., 2012).

Even though the economic significance of diseases caused by *C. graminicola* is clear, little is known about the major defense strategies plants utilize to defend against ALB or ASR. However,

new research suggests that one of the pathways engaged during defense responses against this fungus involves the biosynthesis of oxylipin products (Park, 2011), either acting as molecular signals to activate downstream defense pathways or as molecules with direct antimicrobial activities. The majority of plant oxylipins are the products of the lipoxygenase (LOX) pathway. While they have been shown to have an important role in various physiological functions in plants, they are much better understood in mammals where they are known as eicosanoids, which include several different groups of well-studied hormones such as prostaglandins, thromboxanes, and leukotrienes. Extensive studies of oxylipin functions in humans have resulted in targeting the oxylipin biosynthetic and signal transduction pathways by an estimated 85% of drugs currently in use in the US (Borrego and Kolomiets, 2016). For example, anti-inflammatory drugs such as aspirin have been shown to inhibit the synthesis of prostaglandins (Vane, 1971) and used to relieve fever and pain (Pountos et al., 2011). In plants, oxylipins are less well-studied resulting in the lack of any oxylipin-related knowledge base for improving crop productivity. Nevertheless, some oxylipins such as the phytohormone jasmonic acid (JA) have been implicated in male reproductive development, defense against necrotrophic pathogens, chewing insect herbivores in maize (Yan et al., 2012; Christensen et al., 2013), and response to drought stress (De Domenico et al., 2012). Several oxylipins have potent antimicrobial activities against pathogens (Blée, 2002; Prost et al., 2005). Oxylipins also serve as signaling molecules in interactions between plants and a diverse array of pathogens, including interactions between plants and fungi (Tsitsigiannis et al., 2007; Christensen and Kolomiets, 2011; Borrego and Kolomiets, 2012). Several recent studies showed that oxylipins are necessary for defense against economically-important plant pathogens such as *Aspergillus* spp. (Gao et al., 2009), *Fusarium verticillioides* (Christensen et al., 2014), and *C. coccodes* (Hwang and Hwang, 2010). In addition to their importance in local defense responses, a

recent study suggested that oxylipins are involved in the regulation of beneficial microbe-triggered induced systemic resistance (ISR) against *C. graminicola* (Constantino, Mastouri, Damarwinasis et al., 2013). Collectively, there are over 600 known plant oxylipins that are produced in these pathways and more are regularly discovered each year (Borrego and Kolomiets, 2016).

Oxylipins are products of the enzymatic oxygenation of polyunsaturated fatty acids (PUFA), typically linoleic acid (C18:2) or linolenic acid (C18:3) in plants, by lipoxygenase (LOX) or alpha-dioxygenase. The highly reactive primary products of the LOX reaction, hydroperoxide of fatty acids, are shunted into one of seven enzymatic pathways to produce structurally and functionally diverse oxylipins. These seven different pathways include the pathway branches initiated by the following enzymes: peroxygenase (PXG), divinyl ether synthase (DES), reductase, hydroperoxide lyase (HPL), LOX, epoxy acid synthase (EAS), allene oxide synthase (AOS), and reductase. The AOS branch is responsible for the biosynthesis of the currently best characterized plant oxylipin, the phytohormone JA, which is a defense molecule associated with necrotrophic pathogen defense (Glazebrook, 2005).

Plant LOXs are divided into two groups, depending on which carbon in the fatty acid chain the LOX isoform oxidizes. 9-LOX oxidizes the 9th carbon of the chain and 13-LOX oxidizes the 13th carbon. Out of these two subfamilies, 13-LOXs have been the best-studied as the 13-LOX reaction provides substrate for synthesis of JA. A subgroup of 13-LOXs have also been implicated in the production of green leafy volatiles, GLV, that serve as potent volatile signals in direct and indirect defenses (Christensen et al., 2013).

Across all plants, the 9-LOXs and its oxylipin products are poorly understood. Compared to 13-LOXs, their exact role in defense, growth, and development is relatively obscure. In maize, a 9-LOX, ZmLOX12, is important in defense against *Fusarium verticillioides* by facilitating the

production of JA (Christensen et al., 2014). Another maize 9-LOX, *ZmLOX3*, has been implicated in root defense against nematodes by regulating the production of ethylene (ET), salicylic acid (SA), and JA (Gao et al., 2008). While JA and ET are associated with defense against necrotrophic pathogens, SA is associated with defense against biotrophic pathogens (Glazebrook, 2005). However, the specific biologically active oxylipin molecular species produced by ZmLOX3 or ZmLOX12 that regulate the production of these defense hormones, as of now, are unknown.

ZmLOX4 and ZmLOX5 are paralogs that share 94% amino acid sequence identity to one another (Park et al., 2010) and most likely evolved as a result of whole genome duplication caused by hybridization of ancient maize progenitors. The genomes of the ancestors of maize have gone through duplication several times, including the most recent one 5-12 million years ago, an event that differentiates maize from its close relative sorghum (Schnable et al., 2009). Previously ZmLOX5 was established as a 9-LOX, and due to its close identity to ZmLOX5, ZmLOX4 is also predicted to be a 9-LOX. (Park et al., 2010). *ZmLOX4* is expressed in the roots and apical meristem while *ZmLOX5* is expressed in most aboveground organs upon diverse stresses (Park et al., 2010). These genes are also distinctively induced by different hormones; while both genes are induced by JA, *ZmLOX5*, but not *ZmLOX4*, is induced by abscisic acid (ABA) and SA (Park et al., 2010). Because these genes are nearly identical to one another but have distinct expression patterns, it is hypothesized that *ZmLOX4* and *ZmLOX5* have sub-functionalized during maize evolution following whole-genome duplication. In addition, previous research has shown that *ZmLOX4* and *ZmLOX5* have opposite roles in defense against *C. graminicola* (Park, 2011). Loss-of-function *lox4* mutants are more susceptible to both ASR and ALB, while *lox5* mutants are more resistant. This suggests that *ZmLOX4* is required for maize defense against *C. graminicola* and *ZmLOX5*

facilitates the pathogenicity of the fungus. However, the specific 9-oxylipins produced by these two isoforms are unknown.

To understand the mechanism behind 9-LOXs involvement in maize defense against *C. graminicola*, this project's main objectives were (1) to identify the oxylipins directly synthesized in the pathways initiated by ZmLOX4 and ZmLOX5; and (2) to uncover the role of these two isoforms in the regulation of the biosynthesis of ROS, JA and SA during infection.

2. MATERIALS AND METHODS

2.1 Plant materials

B73 wild type, *lox4-7* and *lox5-3* mutant allele plants were used in this study. Maize plants were placed in a conical tube of MetroMix 360 RSi soil and watered with distilled water every 2-3 days. Plants were left under a light rack under a 16h day length for around a month until they reached V4 developmental stage, where they were inoculated with *C. graminicola* if they are meant for ALB experiments. For ASR experiments, plants were later transplanted into a larger pot inside a greenhouse where they are watered every 2-3 days until their silks start flowering and plants are ready to be inoculated for ASR.

2.2 Genetic crosses

Plants provided by Pioneer were crossed by screening lines with mu transposons for insertions in desired genes. Once an insertion is discovered within an intron through PCR, the plant is crossed with the desired inbred plant (B73) for seven generations (BC7, backcross 7), selected by PCR for the desired insertion, by which the plant is near isogenic to the inbred plant (See primers on Table 2). Each primer pair flanks the predicted insertion, with an insertion resulting in no amplification due to the polymerase activity being disrupted by the hairpin loop of the insertion. 9242 anneals to both sides of the insertion and will only result in amplification with one of the primers of the pair when there a mu insertion is present in the intended area.

Primer name	Sequence
lox4-7F	GTTCCTCAGAAGCATTCTGCCCGAT
lox4-7R	CAAGTTGCCAGACGTGGCCCTCAG
lox5-3F	TGCCGGACCAGTCAAGCCCATAT
lox5-3R	GGCCCCTTCCGGTTCTTCAAGTC
9242	AGAGAAGCCAACGCCAACGCCTCCATTTCGTC

Table 1: Primers used for PCR and genotyping

2.3 *C. graminicola* growth and harvesting of conidia

Strain 1.001 of *C. graminicola* was used. The fungus was cultured on potato dextrose agar (PDA) and maintained under a 12h/12h light/dark cycle under fluorescent light. Conidia were harvested from 2-week-old *C. graminicola* PDA plates by pouring 30ml of sterile water on the agar and scraping off conidia and mycelia using a polystyrene cell spreader, suspension was drawn by using a 1ml pipet and filtering it through five layers of sterilized cheesecloth on top of a funnel, letting it drain into a 50ml conical tube. The conical tube was centrifuged at 3000rpm for three minutes. The liquid was decanted, being careful not to disturb the pellet of conidia at the bottom of the tube. Sterile water was then added again to the conidia, mixed to suspend the pellet, then centrifuged again at 3000rpm for three minutes. The liquid was decanted away again and conidia were resuspended with 20-40ml of distilled water. The proper concentration (10^6 conidia/ml) for inoculation was then calculated using a hemocytometer in a total of 40ml of sterile water with 10 µl of Tween-20 added to the suspension. The suspension was used to inoculate plants within 2 hours of scraping with the cell spreader.

2.4 Treatment of plants with *C. graminicola* conidia and chitin oligosaccharides

For drop inoculation of *C. graminicola* conidia, V4 developmental stage maize plants of B73 wild type, *lox4*-7, and *lox5-3* mutant plants were laid down in trays lined with paper towels and the leaves were taped to be as flat to the surface of the tray as possible. Six 10µl drops of 10^6conidia/ml spore suspension were then pipetted to the inner surface of the leaf, three on the left side and three on the right of the leaf. The paper towels were then moistened with distilled water until saturation. Inoculated plants were then incubated by covering the tray with Press-N-Seal (The Glad Products Company, Oakland, CA, USA) for 24 hours at room temperature. Afterwards, the seals were removed and the plants were returned to the upright position. The leaves were then excised and the lesion area was quantified using the ImageJ (Schneider et al., 2012) software. Statistically significant differences between lesion sizes on different genotypes were calculated using Student's t-test and Tukey's test ($P<0.05$) using JMP PRO 12 (JMP®, Version 12.0.1. SAS Institute Inc., Cary, NC, 1989-2007.).

For experiments with exogenous SA treatment, the hormone was applied to taped leaves by spraying 20ml of 2.5mM concentration salicylic acid onto each genotype group of plant leaves prior to applying conidia suspension for drop inoculation. After spraying, drop inoculation method was continued as described above.

Spray inoculation was conducted by spraying V4 developmental stage maize plants of B73 wild type, *lox4*-7, and *lox5-3* mutant plants that were placed on racks with stakes taped on the side in order to act as support for the roof of the humidity chamber. The plants were then sprayed with an atomizer containing 10^6 conidia/ml suspension containing around 1ml of suspension per plant. Two autoclave bags were cut and taped together in order to make the humidity chamber for the plants and placed on top of the rack using the stakes as support for this 'roof'. Moistened paper

towels were added beforehand in the rack in order to increase humidity before the chamber was closed. The inoculated plants were allowed to incubate for 24 hours in the humidity chamber before the roof was removed and the leaves were excised for further analysis.

Anthracnose stalk rot experiments were done on plants grown in the greenhouse. They were inoculated as the ears of the plant started to flower and the silks are expelled from the ear. The bottom three nodes of the plant were stabbed with a needle, careful to keep the wound size consistent among all plants by using a tool. Conidia was extracted as detailed above. Cotton swabs were dipped in the 10^6 conidia/ml spore suspension of *C. graminicola*, placed on the wound then wrapped around the wound using parafilm. Each node was harvested at the proper incubation period by splitting the wound site lengthwise. Images of the stalk rot lesions were taken with a ruler for scale and quantified using the ImageJ software.

For chitin oligosaccharide treatment experiments, B73 plants were sprayed with 20ml of chitin oligosaccharide solution at 0.1% concentration while the control plants are sprayed with 20ml of sterile water. Plants were separated with cardboard beforehand to make sure the chitin solution aerosols would not touch the control non-chitin-treated plants. The leaves were then excised and harvested at the appropriate timepoints.

For appressoria quantification, infection was done as above, but using *C. graminicola* spore suspension of 10^5 conidia/ml. Chlorophyll from infection sites was removed by submerging leaves in a solution of 75% ethanol+25% glacial acetic acid for 24 hours, changing the solution when needed. The leaves were mounted on the microscope slides using 50% glycerol and images were taken of the whole infection site. These images were then superimposed and appressoria in each infection site were then counted.

2.5 Visualization of hydrogen peroxide

The method was modified from the method described by Liu et al. (Liu and Friesen. 2012; Liu et al., 2012) for larger pieces of maize leaves. Sterile water was first acidified to 3.6 pH with hydrochloric acid to dissolve 3-3' diaminobenzidine (DAB). A 200ml flask containing a concentration of 10mg of DAB/10ml of 3.6 pH water was then dissolved by shaking the flask, covered with aluminum foil to avoid light, in an orbital shaker at 37°C for at least an hour. Afterwards, the liquid was distributed to different 50ml conical tubes, where each maize leaf group was then submerged. With the caps open, the tubes were placed inside a desiccation chamber with a vacuum running for 30 minutes in order to allow the DAB solution to enter the leaves. Afterwards, the tubes were left inside a dark cabinet overnight in order to fully stain hydrogen peroxide (H_2O_2) with DAB. Chlorophyll was removed by submerging leaves in a solution of 75% ethanol+25% glacial acetic acid for 24 hours, changing the solution out when needed. The leaves could be observed under a microscope by mounting leaves in 50% glycerol on a microscope slide and stored long term by submerging it in 50% glycerol solution. An Olympus BX60 microscope was used for light microscopy.

2.6 Fungal mycelium staining

The chlorophyll of the leaves was removed beforehand by submerging the leaves in a solution of 75% ethanol+25% glacial acetic acid for 24h, changing out the solution when needed. Fungal hyphae were stained with 0.01% trypan blue in lactophenol. This concentration of trypan blue powder was mixed in a solution of equal volumes of lactic acid, phenol, and sterile distilled water (1:1:1 by volume). The leaves were then submerged under this solution in a 50ml Falcon tube, then shaken at low speeds overnight in an orbital shaker at room temperature. The staining

solution was then cleared by washing the leaf with 50% glycerol, and then submerging the leaves in 50% glycerol for at least two hours. The leaves could be observed under a microscope by mounting leaves in 50% glycerol on the slide or be stored long term by submerging it in 50% glycerol solution. An Olympus BX60 microscope was used for light microscopy.

2.7 Oxylipin and phytohormone profiling

Plant tissue was ground and weighed to 100 mg +/- 10% per sample and then extracted with 500µl hormone extraction buffer [1-propanol/Water/Concentrated hydrochloric acid (2:1:0.002 by volume)] mixed with hormone standards of d-abscisic acid, d-trans-cinnamic acid, d-jasmonic acid, and d-salicylic acid. Samples were then agitated at 4°C in the dark for 30 minutes. Dichloromethane (500µl) was then added to the samples, followed by agitation at 4°C for another 30 minutes. They were then centrifuged at 15,000 rpm for 5 minutes. The lower organic layer of the solution was then transferred into a glass vial and the solvent was removed using an evaporator under nitrogen gas. After the solvent was dry, the hormones were resolubilized with 150µl of methanol and shaken for one minute. The samples were then moved to 1.5 ml centrifuge tubes and stored overnight in -20°C to allow debris to settle. They were then centrifuged at 15,000 rpm for 4 minutes at 4°C. 100µl of the supernatant was transferred to a clean glass insert placed in a glass vial, careful not to carry any debris, for direct injection into the LC-MS/MS. The detection of hormones utilized methods from Müller and Munné-Bosch (2011) with modifications. For quantification, the column used was an Ascentis Express C-18 Column (3 cm X 2.1 mm, 2.7 um) connected to an API 3200 LC/MS/MS with multiple reaction mentoring (MRM). The mobile phase was 600µl/min consisting of 0.05% acetic acid in water (Solution A) and 0.05% acetic acid in

acetonitrile (Solution B). The gradient consists of (time-%B): 0.3- 1%, 2- 45%, 5-100%, 8-100%, 9-1%, 11-stop.

2.8 DNA extraction

DNA was extracted using the urea extraction buffer method. Around 200mg of tissue was homogenized using Beadbeater homogenizer while suspended in 600µl of Urea extraction buffer. After the samples are homogenized, 600µl of phenol/chloroform solution was added and the solution was vortexed quickly to mix. The tubes were then shaken on a mechanical swing for 15 minutes and observed to make sure the mixture was free-moving. Otherwise, they are vortexed again. Afterwards, the mixture was centrifuged at 14,000rpm at 4°C for 15 minutes. The supernatant, the upper aqueous layer of the solution, was transferred into a different 1.5ml tube to which 60µl of Sodium Acetate (NaOAc) and 700µl of isopropanol were added. The solution was then mixed gently by turning the samples 20-30 times by hand. Afterwards, the solution was centrifuged at 8000rpm for 15 minutes at between 4-25°C. The supernatant liquid was then decanted as waste, careful not to disturb the pellet lodged at the bottom of the tube. The pellet was then washed with 500ul of 70% ethanol, flicked, then centrifuged in a small tabletop centrifuge for 10 seconds. The ethanol was decanted away. This washing step was repeated a total of three times. Afterwards, the remaining ethanol was pipetted out and the tubes were left to air-dry for 15 minutes or until the smell of ethanol went away. The pellets were then dissolved in 150µl of sterile water and stored in 4°C.

2.9 RNA extraction and transcript quantification

Harvested tissues were immediately frozen in liquid N_2 and stored at -80°C. RNA was extracted using the TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA) method. Samples were ground in liquid N_2 using a sterile mortar and pestle. Then 20-100mg of the ground sample was suspended in 1ml of Tri reagent. The samples were then vortexed until mixed well and stored at room temperature for 15 minutes. 200µl of chloroform was then added to the suspension, shaken by hand for 15 seconds, and then stored at room temperature for 15 minutes. They were then centrifuged at 12,000rpm at 4°C for 15 minutes. After centrifuging, the aqueous upper layer was transferred carefully to a 1.5ml tube without disrupting the lower layer. 500µl of isopropanol was added to the supernatant, and then vortexed until it was completely mixed. The solution was then stored at room temperature for 10 minutes and centrifuged at 12,000rpm for 8 minutes in 4-25°C. The supernatant was then removed carefully without disturbing the pellet lodged at the bottom of the tube. The pellet was then washed with 500µl 75% Ethanol mixed with sterile 0.1% diethyl pyrocarbonate (DEPC)-treated water and centrifuged at 8,000rcm for 5 minutes before then removing the ethanol by decanting. The washing and centrifuging step was repeated a total of three times to ensure the cleanest RNA sample possible. The washing ethanol was then removed through pipetting and through air-drying in a fume hood for 15 minutes or until ethanol could not be smelled. The RNA pellet was then dissolved using 50ul of 0.1% DEPC-treated water, incubated for 5 minutes in a 55-60°C water bath, then mixed, and placed on ice for 30 minutes before being stored at -20°C.

Genomic DNA was removed from all RNA samples following the protocol provided by the DNase I, RNase-free kit from Thermo Scientific. RNA concentration was quantified using a Nanodrop 2000 spectrophotometer from Thermo Scientific. RNA was diluted to a concentration

of 125ng/µl, and 8µl of the RNA suspension (a total of 1µg of RNA) was mixed with 1µl of 10x reaction buffer with $MgCl_2$ and 1µl of DNase I, RNase-free (#EN0521), both provided by the kit. The solution was mixed and incubated at 37°C using a heated block for 30 minutes. 1µl of 50mM EDTA was added to each sample and incubated at 65°C for 10 minutes in order to deactivate the DNase I enzyme.

Real time-quantitative PCR was done using the Thermo Scientific Verso SYBR Green 1-Step qRT-PCR ROX Mix kit and conducted in an Applied Biosystems 7500 Fast Real-Time PCR System under the following conditions: 50°C for 15 minutes, 95°C for 15 minutes, and 40 cycles of 95°C for 15s and 54°C for 30s. A melting curve analysis, from 60°C to 95°C with a 0.5°C increment was used to test primers and identify different amplicons. Relative quantification of maize genes was normalized using the housekeeping gene α-tubulin and fold change was

Primer name	**Sequence**
α-tubulin Forward	GCTATCCTGTGATCTGCCCTGA
α-tubulin Reverse	CGCCAAACTTAATAACCCAGTA
ZmLOX4 Forward	TGAGCGGATGGTTTGTAGAT
ZmLOX4 Reverse	ATTATCCAGACGTGGCTCCT
ZmLOX5 Forward	GGGCAGATTGTGTCTCGTAGTA
ZmLOX5 Reverse	ATATTCAAGCGTGGACTCCTCT
BX10 Forward	CAGCAGGTGGTGGTGATAAT
BX10 Reverse	AGCGCCAGACTCACAAAGG

Table 2: Primers used for real-time quantitative PCR

calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). Significant differences were calculated using Tukey's test ($P<0.05$). The primers used are detailed in Table 3.

2.10 Transcriptome analysis through RNA sequencing

B73 inbred, *lox4-7*, and *lox5-3* mutant plants were drop inoculated with 10^6 conidia/ml spore suspension of *C. graminicola.* Leaves were collected at 48 hours after inoculation. Each group used a pool of four different plants infected with *C. graminicola* and ground as one sample with a mortar and pestle.

RNA for the RNA-sequencing experiment was extracted from plant leaves using the protocol provided in RNAeasy Plant Mini Kit from Qiagen. 100mg of frozen hand-ground plant tissue were given 450ul of the provided buffer RLC from the kit mixed with β-mercaptoethanol. The lysate was then transferred into a QIAshredder spin column on a 2ml collection tube, and then centrifuged full speed for 2 minutes. The cleared lysate was transferred to a new microcentrifuge tube without disturbing the debris pellet at the bottom of the collection tube. Half volume of 100% ethanol was then added to the cleared lysate and mixed with pipetting, then immediately transferred to an RNAeasy Mini spin column over a 2ml collection tube. The tubes are then centrifuged for 15s at 10,000 rpm and the flowthrough collected at the collection tube is discarded as the RNA is now bound on the column. 700µl of buffer RW1 was added to the spin column and centrifuged for 15 seconds at 10,000 rpm, and the flowthrough was discarded. 500µl of buffer RPE was added to the spin column and centrifuged for 15 seconds at 10,000 rpm and the flowthrough discarded. This step was repeated a second time. The spin column was then placed in a new collection tube, and then centrifuged at full speed for 1 minute to dry the collection membrane.

The spin column was transferred to a 1.5ml centrifuge tube, 50µl of RNAse-free water was added to the spin column, and centrifuged for 1 minute at 10,000 rpm.

The transcripts were sequenced by the Texas A&M Agrilife research Genomics and Bioinformatics Services with the sequencing machine Illumina HiSeq 2500 (paired end). Samples were multiplexed and run in a single lane to eliminate lane effects. Multiplex sequencing adapters were provided in the Multiplexing sample preparation oligo kit (Ilumina). The reads were aligned to the Ensemble-released maize genome version 3.21 using the aligner program STAR (Dobin et al., 2013) version 2.4.0. Version 3.21 of the Ensembl-released maize reference annotations were used to annotate reads. Read counts were taken using HTSeq (Anders et al., 2015).

Mean difference plots were made using EdgeR on the raw transcript counts. Venn diagrams (Figures 9D and 9E) were done on raw counts on transcripts that are upregulated or downregulated two-fold in mutants compared to WT.

2.11 Proteome analysis through shot-gun proteomics

MudPIT-based shot-gun proteomics analysis was performed following the methods outlined by Zhang et al. (2012) modified for maize proteomic analysis. Proteins were extracted by a plant total protein extraction kit (Sigma-Aldrich, St.Louis, MO) following the manufacturer's protocol. 150 mg of leaf tissue was transferred to a v-bottom freezing vial, 1.5ml methanol with protease inhibitor solution was added, vortexed for 30 seconds, and incubated at -20°C for 5 minutes, and centrifuged at 16,000g for 5 minutes at 4°C. Supernatant was then removed, and the pellet was washed with the methanol solution two more times. The pellets were then air dried and 1.5ml of pre-chilled -20°C acetone was added to the sample. The sample was then vortexed for 30 seconds and incubated at -20°C for 5 minutes and centrifuged at 16,000g for 5 minutes at 4°C. The

supernatant was then removed with a pipette, careful not to disturb the pellet. The pellets were then dried with a SpeedVac to remove residual acetone. Reagent type 4 Working Solution was added to each sample, vortexed to break up the pellet into the working solution, and incubated at room temperature for 15 minutes. The solution was then centrifuged at 16,000g for 30 minutes, and the supernatant, which now contains the protein sample, was pipetted into a clean, labeled tube. The peptides were then processed for LC-MS/MS analysis as described by Zhang et al. (2012).

3. RESULTS

3.1 *ZmLOX4* and *ZmLOX5* differentially respond to *C. graminicola* infection and PAMP elicitation

Previous research has shown that, despite their high homology, *ZmLOX4* and *ZmLOX5* have displayed vastly different expression patterns in diverse maize organs and in response to various stimuli (Park et al., 2010). To determine if *ZmLOX4* and *ZmLOX5* gene expression is also differentially induced in response to *C. graminicola* inoculation, wild-type B73 inbred maize plants were spray-inoculated with a conidial suspension of *C. graminicola* conidia or water as a mock treatment. *ZmLOX4* and *ZmLOX5* transcripts in leaves were quantified by real-time quantitative PCR (qPCR) at 0, 3, 6, 24, and 48 hours post-inoculation (hpi). Figure 1A shows that numerically, *ZmLOX4* transcripts were increased by 3-fold at 48 hpi compared to the mock-treated controls, and maintained numerically higher transcript accumulation compared to mock-treated plants (except at 24 hpi), even if it is not statistically significant. It is important to note that *ZmLOX4* transcripts do not accumulate basally in leaves without challenge (Park et al., 2010). Therefore, this expression pattern suggest that *ZmLOX4* is induced by *C. graminicola* at the approximate timing for the switch between biotrophic and necrotrophic phases in disease development (Mims and Vaillancourt, 2002). *ZmLOX5*, conversely maintains numerically lower levels of transcript accumulation in response to *C. graminicola* conidia compared to mock-treated plants (Fig. 1B). Interestingly, corroborating previous expression analyses by Park et al. (2010), *ZmLOX5* displayed induction in response to the water spray, indicating that this gene is touch-inducible. Suppression of ZmLOX5 in response to infection is consistent with the reported role of this gene as a susceptibility gene (Park, 2011).

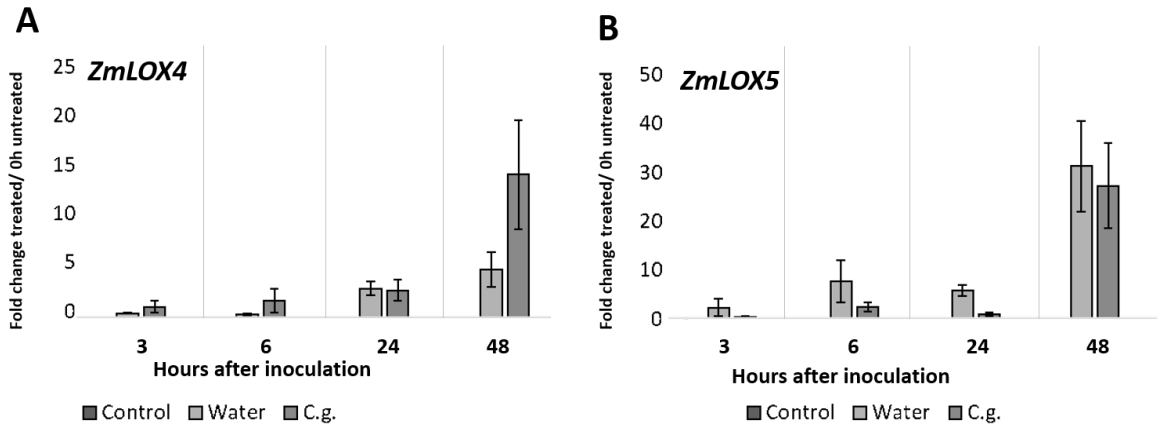

Fig. 1: *ZmLOX4* and *ZmLOX5* show differential gene expression in response to infection by *C. graminicola*. B73 plants were sprayed with spore suspension of *C. graminicola* or water and leaves were harvested at 3, 6, 12, 24, 48 hours post-inoculation (hpi) and transcripts were quantified using qPCR. (A) Fold change of *ZmLOX4* in leaves treated with water or *C. graminicola*, compared to untreated leaves. (B) Fold change of *ZmLOX5* in treated with water or *C. graminicola*, compared to untreated leaves. Error bars represent means ± standard error from at least three biological replicates (*P<0.05, Student's t-test of different treatments within time points).

One of the unexpected findings from this study is that increased transcript accumulation of *ZmLOX5* was observed at 48 hours after treatment in plants treated with water or *C. graminicola* (Fig. 1B). This can be explained by the findings of Constantino (2017) studying pathogen-induced plant volatiles (PIPV). Twenty-four hours after inoculation, plants were removed from their humidity chambers and placed within nine feet of each other, allowing volatiles from *C. graminicola*-treated plants to interact with mock-treated plants. *ZmLOX5* is induced by PIPVs from *C. graminicola*-treated plants (Constantino, 2017), which explains the substantial induction of *ZmLOX5* in mock-treated plants at 48hpi (Fig. 1B).

Although no statistically significant differences of *ZmLOX4* expression were detected between the group treated with water and the group treated with *C. graminicola* conidia, except for 24 hpi, *ZmLOX4* showed a consistent trend of higher numerical expression in leaves treated with *C. graminicola*, even at the very early time point of 3 hpi (Fig. 1A). This suggests that *ZmLOX4* may be induced by fungal pathogen-associated molecular patterns (PAMPs) secreted by

C. graminicola. This hypothesis was tested by the analyses of expression in response to chitin treatment. *C. graminicola* has a cell wall composed largely of chitin. Chitin has been extensively used to study how plants respond to fungal PAMPs (de Jonge et al., 2010; Eckardt, 2008). B73 inbred leaves were treated by spraying with sterile water or 0.1% chitin oligosaccharide solution, and expression of *ZmLOX4* and *ZmLOX5* was measured at 15, 30, 60, and 120 min after treatment. Fig. 2A shows that *ZmLOX4* has a higher numerical transcript accumulation in plants treated with chitin at 30 min compared to mock-treated plants, but only transiently, as its transcription levels returned to basal levels at 60 min after treatment, which suggests that the gene might be involved in PAMP-triggered immunity (PTI), which is defined as an activation of innate immune response upon detection of PAMPs (Thomma et al., 2011). *ZmLOX5* is not significantly induced by chitin,

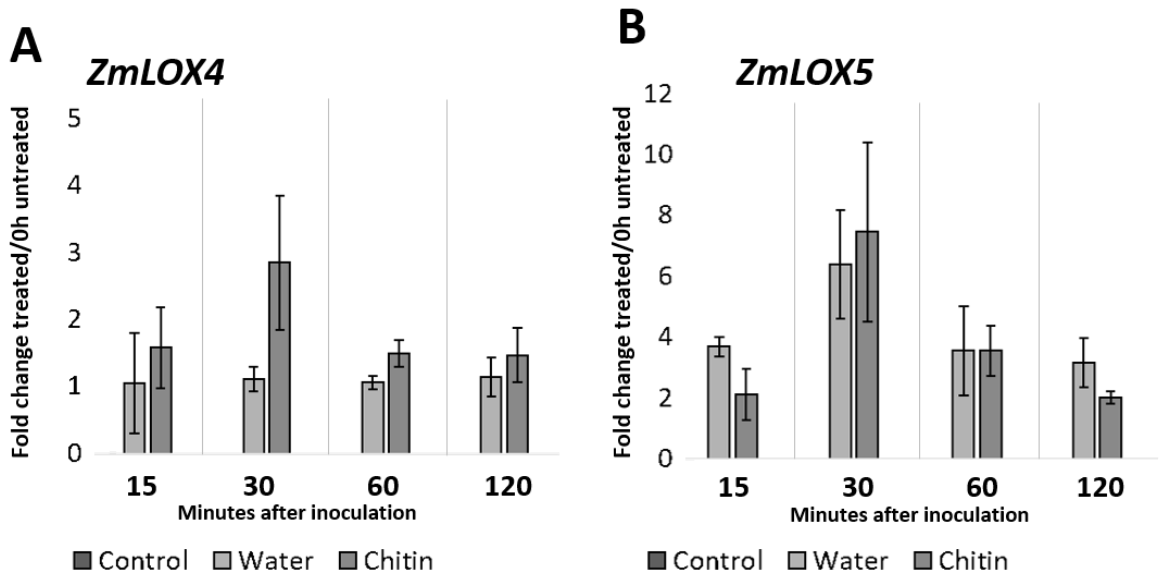

Fig. 2: *ZmLOX4* and *ZmLOX5* show differential gene expression in response to spray treatment of chitin oligosaccharide solution or water. B73 plants were sprayed with 20 ml of chitin oligosaccharide solution or water and leaves were harvested at 15, 30, 60, and 120 minutes post-inoculation (mpi) and transcripts were quantified using quantitative real-time PCR. (A) Fold change of *ZmLOX4* in leaves treated with water or with chitin solution, compared with untreated leaves. (B) Fold change of *ZmLOX5* in leaves treated with water or with chitin solution, compared with untreated leaves. Gene expression was normalized using the α-tubulin housekeeping gene as an endogenous control. Error bars represent means $\pm$ standard error from at least three biological replicates ($P<0.05$, Student's t-test of different treatments within time points).

but is induced as fast as 15 minutes after treatments with both mock and chitin solution (Fig. 2B). This suggests that *ZmLOX5* is either induced by water or touch. Previous research has shown that *ZmLOX5* is responsive to touch and is an important gene for insect defense, particularly fall armyworm (Park, 2011). *ZmLOX5* touch-responsiveness may be important in detecting incoming insect herbivores, and numerous studies in different plants have shown that touch treatments can induce resistance that can protect the plant against pests (Chehab et al., 2012; Markovic et al., 2014; Markovic et al., 2016).

3.2 The *ZmLOX4* promoter contains pathogen-inducible cis-acting elements while the *ZmLOX5* promoter contains elements associated with JA- and SA-inducibility

Due to differential expression of the two genes in response to diverse hormone treatments (Park, 2011) and infection (Fig. 1), it is hypothesized that the promoter regions between these two genes may be responsible for this differential expression. The promoter regions of *ZmLOX4* and *ZmLOX5* were analyzed with PLACE Signal Scan Search (http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999) and compared to a list of 177 known cis-acting elements in plants to identify cis-elements that are differentially predicted between the two sequences. Annotations for these cis-acting elements were found in PlantPAN2.0 (http://plantpan2.itps.ncku.edu.tw/; Chow et al., 2016). Among the most divergent promoter elements, listed on Table 1, *ZmLOX4* promoter region contains a CACGTG motif that have been shown to be important for many defense-related genes (Hudson and Quail, 2003; Menkens et al., 1995). The presence of this cis-acting element matches its hypothesized role as a defense gene against infection by *C. graminicola* (Park, 2011). Meanwhile, the *ZmLOX5* promoter region contains the JASE1ATOPR1 motif, which is upregulated during senescence and JA (He and Gan, 2001), and a TCA1 motif, which is related to

SA-inducible expression of defense genes (Goldsbrough et al., 1993), supporting previous findings that JA, SA and ABA induce *ZmLOX5* (Park et al., 2010).

Gene	Cis-acting element	Potential induction
ZmLOX4	CATATGGMSAUR	Auxin
	ACGTABREMOTIFA2OSEM	ABA
	CACGTG motif	Involved in expression of defense-related genes
ZmLOX5	JASE1ATOPR1	Senescence and JA
	T/GBOXATPIN2	Jasmonates
	TCA1 motif	SA
	DRE1COREZMRAB17	ABA

Table 3: Selected cis-acting elements that are potentially responsive to phytohormones or important in plant defense predicted within 1kb upstream of the *ZmLOX4* and *ZmLOX5* transcription start sites exclusively in one or the other.

3.3 *ZmLOX4* is essential for maize defense against *C. graminicola* while *ZmLOX5* facilitates pathogenicity

Previous preliminary research has shown that *lox4* mutant plants are more susceptible to *C. graminicola*, while *lox5* plants are more resistant (Park, 2011), which, along with the expression patterns found in Figures 1 and 2, suggest that *ZmLOX4* is involved in maize defense against fungal pathogens, while *ZmLOX5* facilitates pathogenicity. Previous research by Park (2011) into the virulence of *C. graminicola* in *lox4* and *lox5* mutants were conducted on plants that were in the

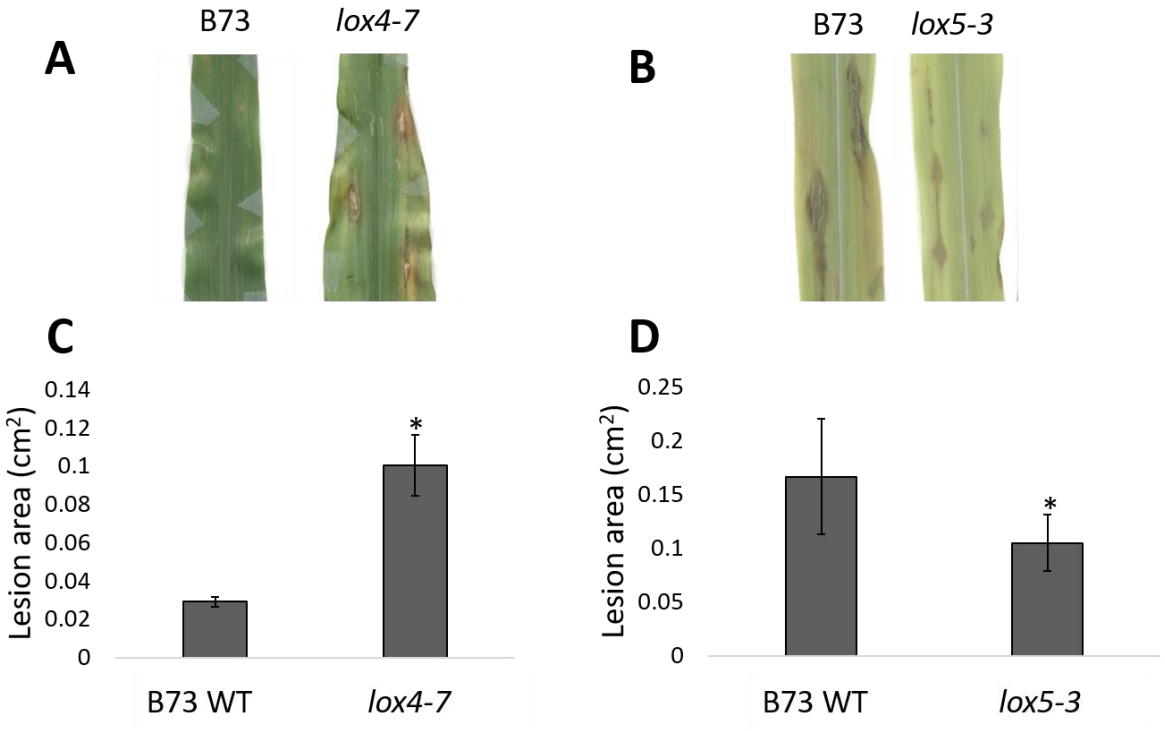

Fig. 3: ZmLOX4 is essential for plant resistance to ALB while ZmLOX5 facilitates ALB disease. Leaves of WT, *lox4-7* and *lox5-3* maize plants were drop-inoculated with *C. graminicola* spore suspension and harvested 3-4 days post inoculation (dpi). Lesion area was measured using ImageJ. (A) ALB disease symptoms on WT and *lox4-7* mutants at 3 dpi, (B) ALB disease symptoms on WT and *lox5-3* mutants at 4 dpi, (C) Comparison of ALB lesion areas between WT and *lox4-7* mutants, (D) Comparison of ALB lesion area between WT and *lox5-3* mutants. Error bars represent means $\pm$ standard error from three biological replicates (*P<0.05 Student's t-test).

earlier genetic stage, i.e. BC4 stage. Since that time, mutant near-isogenic lines at the BC7 genetic stage, which are nearly genetically identical to the B73 inbred, were developed. For this reason, the virulence assays were confirmed with advanced genetic tools for both ALB and ASR. For ALB, B73 wild type (hereafter named WT), *lox4-7*, and *lox5-3* mutant alleles were drop-inoculated with a *C. graminicola* spore suspension, and the infection was allowed to proceed for 3 to 4 days. Virulence of the fungus was assessed by measuring the resulting lesions area using ImageJ (Schneider et al., 2012). ALB lesions on *lox4-7* mutants were significantly larger than on WT (Fig. 3A, C). In contrast, lesions on *lox5-3* mutants were significantly smaller (Fig. 3B, D). This

confirms the results by Park (2011) and showed that *ZmLOX4* is required for maize resistance to ALB, while *ZmLOX5* facilitates pathogenicity.

An experiment was also conducted to test the virulence of *C. graminicola* on *lox4-7* and *lox5-3* plants with regard to ASR. WT, *lox4-7*, and *lox5-3* mutant plants were grown in a greenhouse and inoculated via stalk wounding. Similar to the levels of resistance observed in the

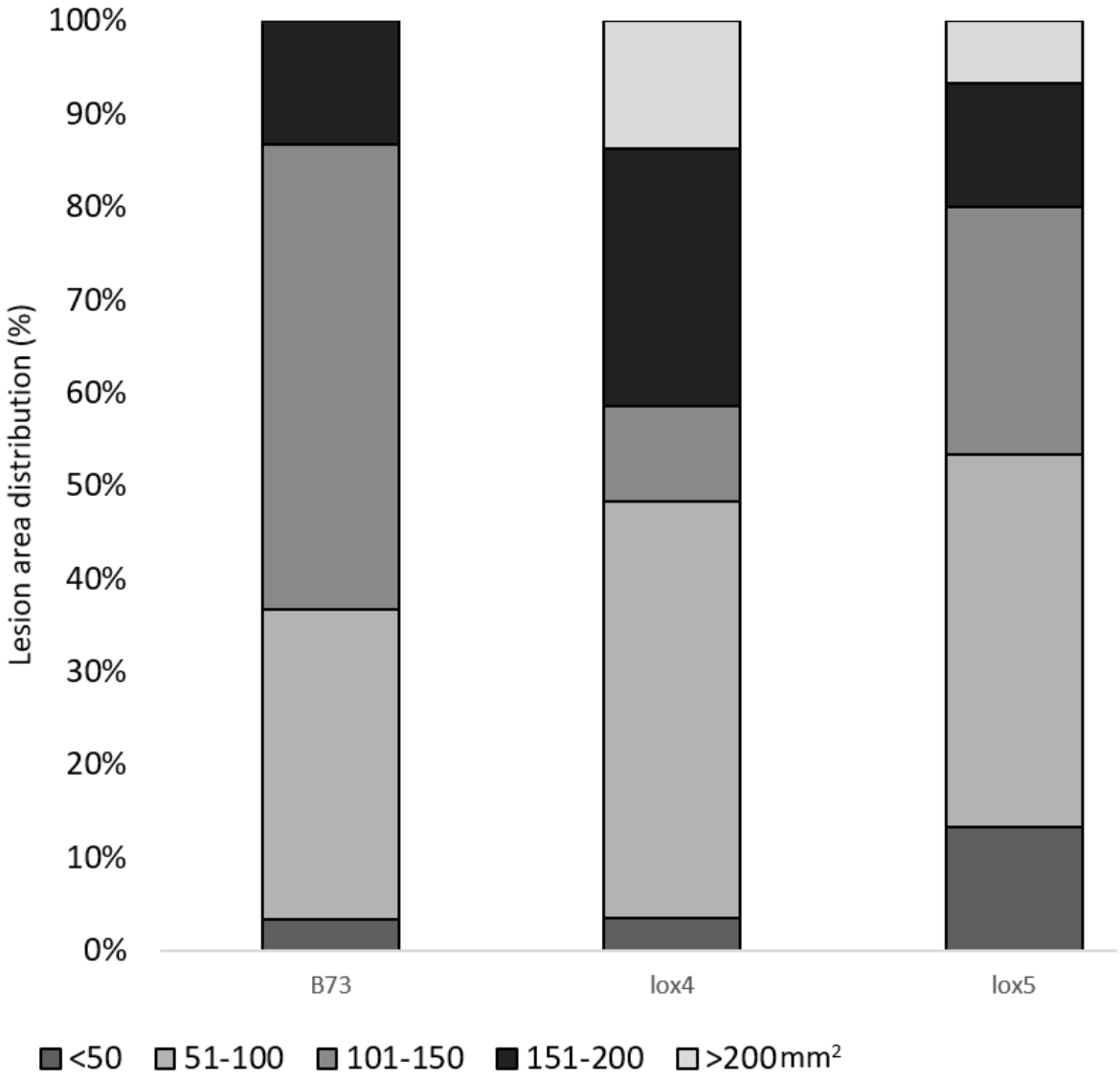

Fig. 4: ZmLOX4 is essential for plant resistance to ASR while ZmLOX5 facilitates ASR disease. Distribution of lesion sizes among samples of ASR on stalks of WT, *lox4-7* and *lox5-3* maize plants 8 days after anthracnose stalk rot inoculation. At least four biological replicates were used for each group.

ALB experiments, *lox4-7* mutants were more susceptible to ASR compared to WT, as over 40% of examined lesions on *lox4-7* mutants were over 150 mm^2 compared to only 12% in WT. (Fig. 4). Meanwhile *lox5-3* mutants were more resistant to ASR compared to WT, as more than 10% of lesions in *lox5-3* are smaller than 50 mm^2 compared to less than 3% in B73 plants (Fig. 4). These results suggest that, as in leaves, *lox4-7* plants were more susceptible to *C. graminicola* compared to the wild type, while *lox5-3* plants are more resistant.

3.4 ZmLOX4 and ZmLOX5 regulate the production of oxylipins in several branches of the LOX pathway

To identify specific oxylipins produced by the two isoforms, over 60 oxylipin molecular species were quantified after infection in the two mutants and compared to WT. LOXs such as *ZmLOX4* and *ZmLOX5* catalyze enzymatic reactions that result in the production of specific oxylipins that can act either as hormone-like signals to induce plant defense or as metabolites with direct antimicrobial activities. Signaling activity by oxylipins is postulated to regulate downstream defense hormones and defense-related metabolites. The LOX enzyme family produces an array of diverse oxylipins that fulfill different functions even when these diverse pathways mediated by individual LOX isoforms utilize a single fatty acid hydroperoxide substrate. I hypothesized that ZmLOX4 and ZmLOX5 enzyme isoforms produce specific 9-oxylipins that initiate either defense responses in the case of ZmLOX4 or facilitate fungal pathogenicity in the case of ZmLOX5-derived oxylipins. Our approach was to profile the oxylipins and phytohormones produced by B73 wild type, *lox4-7* and *lox5-3* mutants in response to *C. graminicola* to identify 9-oxylipins that accumulate to lower levels or missing in the mutants. Plants were spray-inoculated and leaves were

harvested at 0, 3, 6, 24, and 48 hours after inoculation. The oxylipins were profiled using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Unexpectedly, no single individual 9-LOX-derived oxylipin assayed in this experiment displayed significantly lower basal accumulation in either *lox4-7* or *lox5*-3 mutants compared to B73. Figure 5 displays total oxylipins produced by the individual LOX pathway branches. In the 9-AOS pathway, I combined the concentrations for 10-oxo-11-phytoenoic acid (10-OPEA), 9OH-10KOD, and 9OH-10KOM levels (Fig. 5A). For the 9-EAS branch, concentrations for 9(S),12(S),13(S)-trihydroxy-10(E),15(Z)-octadecatrienoic acid (9,10,13-THOM) and

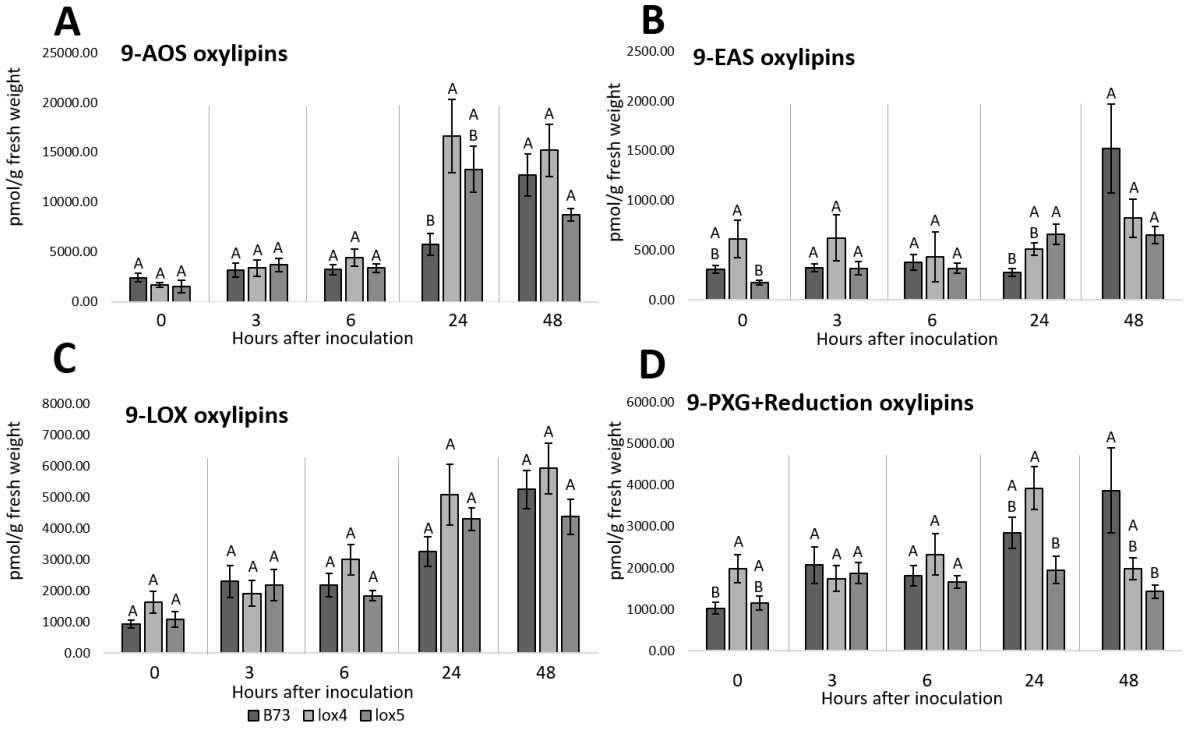

Fig. 5: 9-oxylipins produced by WT, *lox4-7*, and *lox5-3* mutants infected with *C. graminicola*, separated by pathway. B73 wild type, *lox4-7*, and *lox5-3* mutant plants were drop inoculated with *C. graminicola* spore suspension and the leaves were excised at 0, 3, 6, 24, and 48 hours after inoculation. (A) 9-oxylipins produced in the 9-AOS pathway in response to *C. graminicola*, (B) 9-oxylipins produced in the 9-EAS pathway in response to *C. graminicola*, (C) 9-oxylipins produced in the 9-LOX pathway in response to *C. graminicola*, (D) 9-oxylipins produced in the 9-peroxygenase and reduction pathways in response to *C. graminicola*. Error bars represent means ± standard error from at least three biological replicates. ($P<0.05$, Tukey's HSD of different treatments within time points)

9(S),12(S),13(S)-Trihydroxy-10(E),15(Z)-octadecadienoic acid (9,12,13-THOM) were combined (Fig. 5B). For the 9-LOX dehydration branch, 9-keto-10(E),12(Z)-octadecadienoic acid (9KOD) and 9-keto-10(E),12(Z),15(Z)-octadecatrienoic acid (9KOT) were summed (Fig. 5C). Since peroxygenase and reduction pathways both produce 9-hydroxyoctadecadi(tri)enoic acid (9HOD) and 9(S)-hydroxy-10(E),12(Z),15(Z)-octadecatrienoic acid (9HOT), both were merged by combining concentrations for 9HOD, 9HOT, and (±)-cis-9,10-epoxyoctadecanoic acid (9,10 EPOM) (Fig. 5D). This analysis showed that *lox4-7* produces a statistically significantly higher amount of oxylipins in the 9-AOS pathway at 24 hpi, though a numerical increase in these oxylipins in *lox5-3* mutant can also be seen (Fig. 5A). Meanwhile, *lox5-3* mutants show statistically significantly higher accumulation of 9-EAS oxylipins at 24 hpi, though likewise, a numerical increase can also be seen in *lox4-7* mutants at the same time point (Fig. 5B). Though not statistically significant, both mutants also display a lower accumulation of these oxylipins at 48 hpi (Fig. 5B). Neither *lox4-7* and *lox5-3* produce statistically significantly different amounts of oxylipins in the 9-LOX dehydration pathway (Fig. 5C). In the 9-PXG and reductase pathways, *lox4-7* mutants produced significantly higher amounts of oxylipins compared to WT basally, while they are produced at significantly lower amounts in *lox5-3* mutants (Fig. 5D). However, in both cases these oxylipins are accumulated the same way on both mutants compared to in WT plants (higher accumulation basally and lower accumulation at 48 hpi) (Fig. 5D). While this experiment did not reveal the exact oxylipin produced specifically by ZmLOX4 or ZmLOX5 that are relevant to defense against *C. graminicola*, the results suggest that these two yet-unidentified oxylipins suppress the production of 9-AOS-derived molecules and regulate the production of 9-EAS and 9-PXG and/or reductase-derived molecules in response to *C. graminicola* infection.

3.5 Loss-of-function *lox5* mutants produce higher amounts of SA and JA-Ile in response to *C. graminicola*

Because oxylipins are implicated as understudied signals that regulate accumulation of defense hormones and defense-related metabolites, I hypothesized that ZmLOX4 and ZmLOX5 may regulate the biosynthesis of defense-related phytohormones that positively or negatively impact host defense against *C. graminicola*. Phytohormone analysis revealed that, compared to WT, both SA and JA-Ile were produced to significantly higher levels in *lox5-3* mutants but to lower levels in *lox4-7* mutants. In WT, SA was induced at 3 hpi, but it returned to near basal levels at every timepoint afterward (Fig. 6A). In *lox4-7* mutants, SA levels were basally low, and maintained low levels compared to both WT and *lox5-3* throughout the infection process (Fig. 6A). SA levels in *lox5-3* was induced 6 hpi and maintained high levels of accumulation throughout the tested timepoints (Fig. 6A). SA accumulated to higher levels in *lox5-3* mutants at 24 hpi compared to WT and *lox4-7* mutant, and a similar trend was observed as well at 6 and 48 hpi (Fig. 6A). JA-Ile in WT was slightly induced after inoculation and except for from 6 to 24 hpi, its levels continued to increase throughout the infection process (Fig. 6B). In *lox4-7* mutants, JA-Ile was induced after inoculation similar to WT, but decreased at 48 hpi while WT displayed the highest levels following infection at this timepoint (Fig. 6B). JA-Ile in *lox5-3* was also induced after inoculation, and to very high levels at 6 hpi. When accumulation levels between the genotypes in each time point were compared, JA-Ile was only found to be significantly increased in *lox5-3* mutants compared to the other genotypes at 6 hpi (Fig. 6B). The high levels of SA and JA-ile in *lox5-3* mutants is the potential explanation underlying the increased resistance of the *lox5-3* mutants compared to WT. Conversely, the lower basal and the lack of pathogen-inducible levels of SA in *lox4-7* mutants is the likely cause for the decreased resistance observed in *lox4-7* mutants.

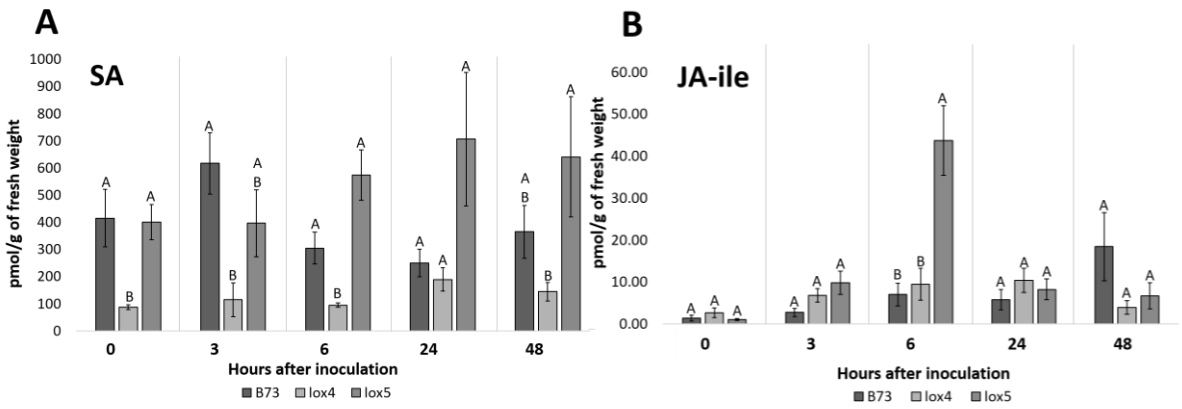

Fig. 6: SA and JA-Ile produced by WT, *lox4-7*, and *lox5-3* mutants infected with *C. graminicola.* B73 WT, *lox4-7*, and *lox5-3* mutant plants were drop inoculated with *C. graminicola* spore suspension and the leaves are excised at 0, 3, 6, 24, and 48 hours after inoculation. (A) SA produced in various time points in response to *C. graminicola* infection. (B) JA-Ile produced in various time points in response to *C. graminicola* infection. Error bars represent ± standard error from at least three biological replicates. ($P<0.05$, Tukey's HSD of different treatments within time points)

3.6 Salicylic acid confers resistance to wild type and *lox4* loss-of-function mutant plants but not to *lox5* mutants

SA is a plant hormone important in plant defense against biotrophic and hemibiotrophic pathogens including *C. graminicola*. In order to test the hypothesis that low or high levels of SA in *lox4* and *lox5* mutants, respectively, are a major mechanism explaining their contrasting resistance levels against *C. graminicola*, the effect of exogenous SA application of the resistance of the two mutants was tested. For this, WT, *lox4-7*, and *lox5-3* mutant plants were treated by spraying the plants with 2.5 mM SA immediately before drop-inoculation with *C. graminicola* spore suspension.

SA treatments increased resistance to *C. graminicola* for WT and *lox4-7* mutant, as shown by significantly reduced area of the necrotic lesions compared to water controls (Fig. 7). WT plants treated with SA were able to mount resistance levels comparable to those found in *lox5-3* mutant.

However, even though *lox4-7* plants were more resistant after SA treatment compared to control, they did not reach the level of resistance displayed by *lox5-3* (Fig. 7). SA treatments of *lox5-3* plants did not provide any increased resistance to *C. graminicola* (Fig. 7), suggesting that the levels of SA in the *lox5-3* mutant is sufficient to effectively defend against *C. graminicola*.

These results suggest that differential SA accumulation in the three genotypes may explain their phenotypic differences in terms of resistance to *C. graminicola*. To the best of my knowledge such a role of 9-oxylipins in the regulation of SA has not been reported before and warrants further investigation.

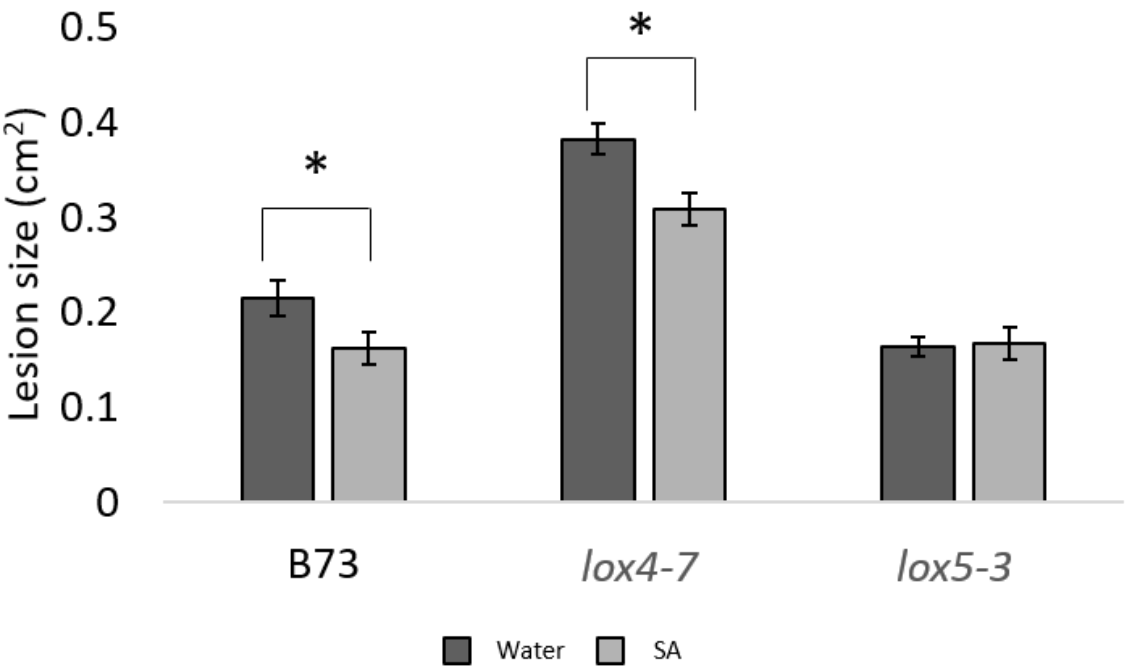

Fig. 7: Salicylic acid treatment increases resistance of maize plants against *C. graminicola* but not on *lox5* mutants. ASR disease progression of *C. graminicola* on B73 WT, *lox4-7* mutant, and *lox5-3* mutant plants. Leaves of maize plants in V4 developmental stage were treated with water or 2.5mM salicylic acid and immediately drop inoculated with *C. graminicola* on the third leaves. Inoculated third leaves were harvested 4 days post inoculation (dpi). ALB lesion area was measured between B73 WT, *lox4-7*, and *lox5-3* mutants treated with water or 2.5mM salicylic acid using the ImageJ software. Error bars represent $\pm$ standard error from at least three biological replicates (*P<0.05 Student's t-test).

3.7 Genes involved in SA and benzoxazinoid biosynthesis, reactive oxygen species scavenging, and protein tyrosine kinases are differentially expressed in *lox4* and *lox5* mutants

In order to identify the genes that are differentially regulated by ZmLOX4- and ZmLOX5-derived oxylipins in response to *C. graminicola*, WT, *lox4-7,* and *lox5-3* mutants were inoculated with *C. graminicola* and global transcriptome analyses on these samples were performed using RNA-sequencing (RNA-seq) at 48 hpi. Genes that were found to be highly expressed in the susceptible *lox4-7* mutant and under-expressed in *lox5-3* mutants compared to WT were considered as potential factors associated with susceptibility against *C. graminicola*, and genes showing the opposite expression pattern were considered associated with defense. Other expression patterns were also sought after to determine potential pathways differentially regulated by *ZmLOX4* and *ZmLOX5*.

RNA-seq analysis revealed an *ISOCHORISMATE SYNTHASE1* (*ICS1*) homolog gene (GRMZM2G022837) that is upregulated in the *lox5-3* mutant plants compared to the other genotypes (Fig. 8A). *ICS1* synthesizes isochorismate, an immediate precursor of SA (Wildermuth et al., 2001). SA synthesized from this pathway has been shown to be required for plant defense responses in *Arabidopsis* (Wildermuth et al., 2001). It is possible that overexpression of this ICS homolog in the *lox5-3* mutant may explain the increased SA-mediated defense observed.

Another gene identified by RNAseq analysis was *CATALASE3* (*CAT3*) (GRMZM2G079348) that encodes for an enzyme which scavenges hydrogen peroxide to detoxify

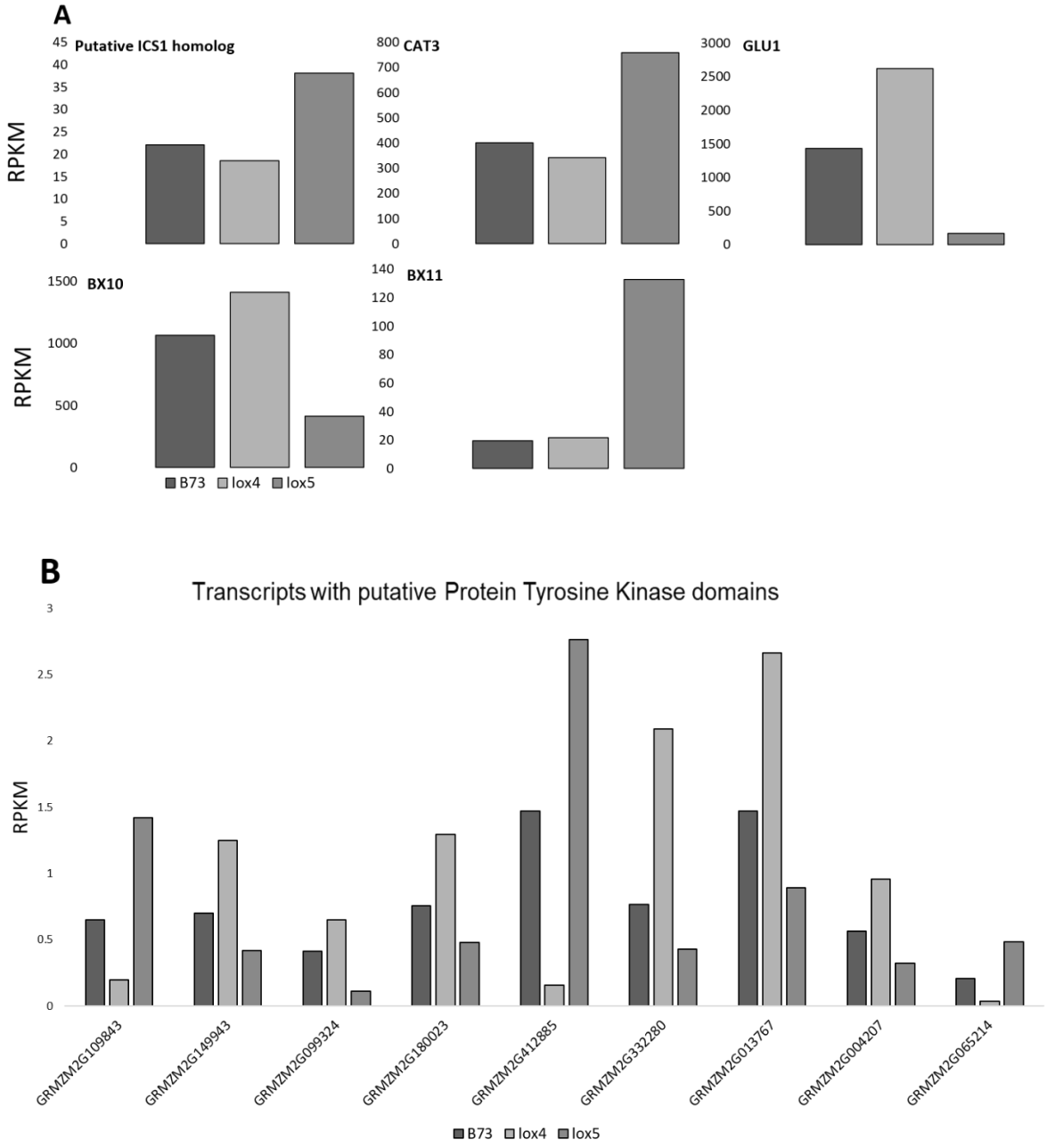

Fig. 8: Genes involved in SA production, ROS scavenging, benzoxizanoid development, and tyrosine kinase domains are differentially regulated in WT, *lox4-7*, and *lox5-3* plants. Samples were taken at 48 hpi(A) Transcripts of ICS1, CAT3, GLU1, BX10, and BX11 that are differentially expressed in the three genotypes. (B) Transcripts containing putative protein tyrosine kinase functional domains that are differentially expressed in *lox4-7* and *lox5-3* mutants compared to WT.

the harmful effect of the reactive oxygen species (ROS) (Conrath et al., 1995). This gene was expressed to higher levels in *lox5-3* mutants compared to either WT or *lox4-7*. Catalase activity is suppressed by SA to increase ROS accumulation.

Two genes of the benzoxazinoid pathway, *BENZOXAZINONE SYNTHESIS10* (*BX10*) (GRMZM2G311036) and *BENZOXAZINONE SYNTHESIS11* (*BX11*) (GRMZM2G336824), were also found to be differentially expressed in *lox4-7* and *lox5-3* mutants (Fig. 8A). Benzoxazinoids are secondary metabolites that have been shown to confer resistance to a variety of pests and pathogens (Meihls et al., 2013). In maize seedlings, the prominent benzoxazinoid is 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Meihls et al., 2013). The BX genes in the DIMBOA pathway, specifically, *BX10* and *BX11* are enzymes that convert 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (DIMBOA-Glc) to 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one glucoside (HDMBOA-Glc) (Meihls et al., 2013; Handrick et al., 2016). DIMBOA-Glc has been shown to promote callose deposition, and its conversion to HDMBOA-Glc by BX10 and BX11 reduces callose deposition in response to aphids (Meihls et al., 2013). *β-GLUCOSIDASE1* (*GLU1*) (GRMZM2G016890) was found to be expressed at low levels in *lox5-3* mutants (Fig. 8A) compared to WT. GLU1 has a role in the synthesis of DIMBOA using DIMBOA-Glc (Meihls et al., 2013) and apoplast infiltration of DIMBOA in maize is known to mimic chitosan-induced callose deposition (Ahmad et al., 2011). Taken together, these data prompt the speculation that DIMBOA-Glc and callose deposition are factors for maize defense against *C. graminicola*. Unfortunately, multiple attempts to directly observe callose deposition in infected leaves was unsuccessful.

A group of proteins containing putative tyrosine kinase functional domains were discovered among the transcripts differentially expressed among the three genotypes. (Fig. 8B).

Tyrosine kinases typically add a phosphate group from ATP to a protein at tyrosine residues (Neel and Tonks, 1997). Many genes with this putative tyrosine kinase domain were either overexpressed in *lox4-7* mutants and under expressed in *lox5-3* mutants compared to B73 WT or vice versa. This

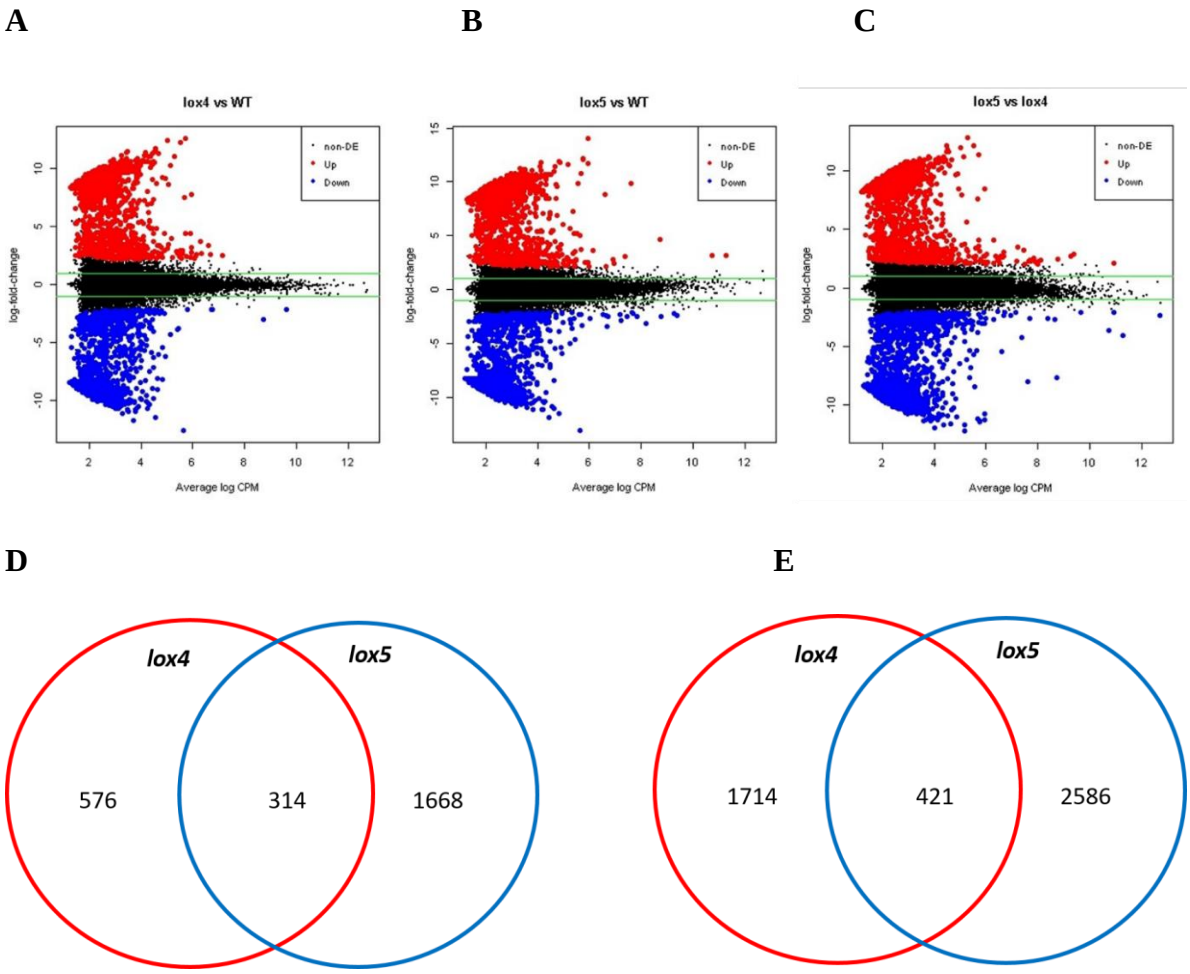

Fig. 9 Mean-difference plot showing transcripts estimated to possess log-2 fold-change between the compared samples. Comparison of transcript expression patterns in WT, *lox4-7*, and *lox5-3* mutants. (A) Plot comparing means between *lox4-7* mutant and WT. (B) Plot comparing means between *lox5-3* and WT. (C) Plot comparing means between *lox5-3* and *lox4-7*. (D) Venn diagram of transcripts which are upregulated in *lox4-7* compared to WT (red circle) and in *lox5-3* compared to WT (blue circle). (E) Venn diagram of transcripts which are downregulated in *lox4-7* compared to WT (red circle) and in *lox5-3* compared to WT (blue circle). In (A), (B), and (C), Red dots represent transcripts that are overexpressed in the first item in the comparison. Blue dots represent transcripts that are underexpressed in the first item in the comparison. Black dots represent genes which are not different between the two genotypes. Mean-difference plots were made using EdgeR. Numbers in (D) represent transcripts which are expressed at least at a 2-fold increase in the mutant genotypes compared to WT and (E) represent transcripts which are expressed at least at a 2-fold decrease in the mutant genotypes compared to WT.

indicates that ZmLOX4 and ZmLOX5 are may be important in the regulation of protein phosphorylation by as yet-unknown mechanisms.

To estimate the number of genes differentially regulated by LOX4 and LOX5, a genewise negative binomial generalized linear model with estimated dispersion was produced with EdgeR (Robinson et al., 2010; McCarthy et al., 2012). This analysis estimates that compared to WT, 1043 transcripts are upregulated in *lox4* and 1348 transcripts are downregulated (Fig. 9A). Compared to WT, *lox5-3* had 1115 transcripts upregulated in and 1447 are downregulated (Fig. 9B). Comparisons between *lox4-7* and *lox5-3* estimate that 1527 transcripts were upregulated in *lox4-7* mutants compared to *lox5-3* and 1495 transcripts were upregulated in *lox5-3* compared to in *lox4-7* (Fig. 9C). Figures 9D and 9E also show that different genes are upregulated and downregulated by disruptions of ZmLOX4 and ZmLOX5. While 314 transcripts are upregulated in both *lox4-7* and *lox5-3* mutants, 576 transcripts are upregulated only in *lox4-7* mutants and 1668 transcripts are only upregulated in *lox5-3* (Fig. 9D). Meanwhile, 421 transcripts are downregulated in both mutants, while 1714 transcripts are downregulated only in *lox4-7* mutants and 2586 transcripts are downregulated in *lox5-3* mutants (Fig. 9E). These changes in global expression reveal the extent of the transcriptional reprogramming caused by the disruption of the two genes.

3.8 BX10 is basally upregulated in *lox4* mutants

To validate the observations from RNA-seq analysis, qPCR was performed to quantify selected genes found to be differentially expressed in B73 wild type, *lox4-7*, and *lox5-3*. One of the candidate genes was *BX10*, which was differentially expressed in the RNAseq experiment (Fig. 10). Interestingly, this gene is over-expressed in *lox4* mutant, but suppressed in the *lox5* mutant in unchallenged leaves, a pattern repeated in the infected leaves at 72 hpi.

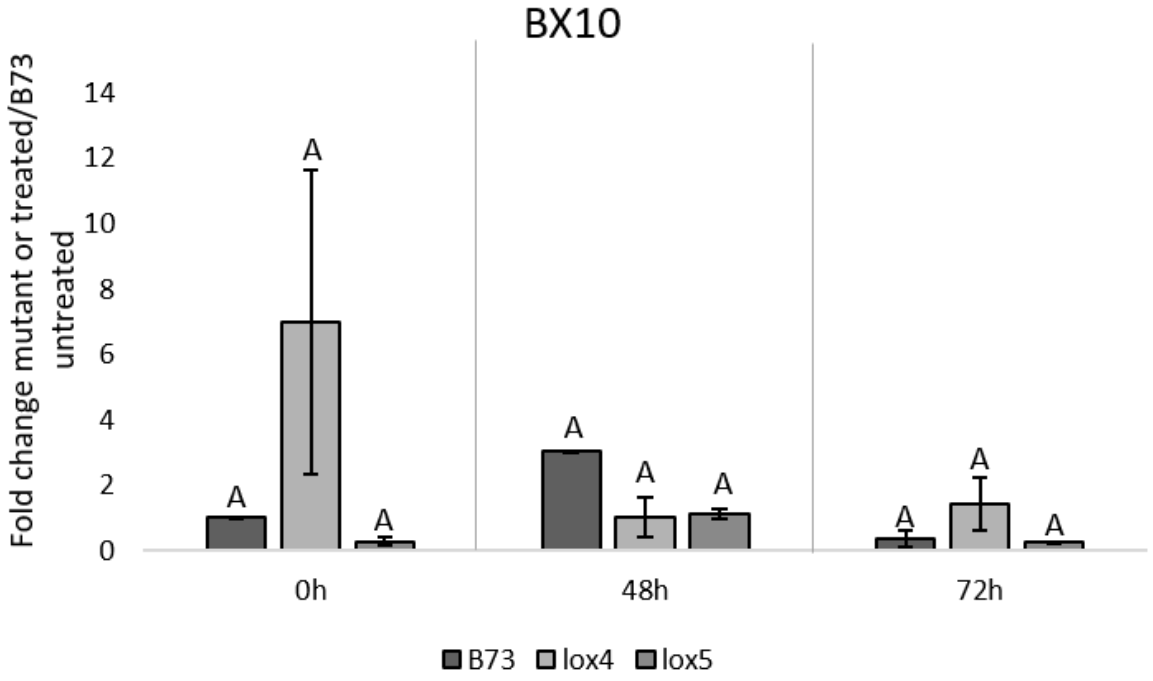

Fig. 10 BX10 is expressed at a high level basally in *lox4-7* mutants. Error bars represent means ± standard error from three biological replicates (P<0.05 Tukey's test on different treatments within time points).

3.9 Shot-gun proteome analysis reveals the upregulation of putative xylanase inhibitor in *lox5* mutants and the differential accumulation of protein involved in ROS accumulation and scavenging

Proteomic analysis was used to identify differentially expressed proteins that are regulated by *ZmLOX4*- and *ZmLOX5*-mediated pathways in response to *C. graminicola*. Protein accumulation in infected leaf tissues of WT, *lox4-7*, and *lox5-3* was measured by shot-gun proteomic analysis as described by Zhang et al. (2012).

A protein with a putative xylanase inhibitor functional domain (GRMZM2G053206) accumulated at higher levels in *lox5* mutant compared to WT and *lox4* (Fig. 11). Xylanase is fungal

cell wall degrading enzyme (Collins et al., 2005), and thus reasonable to hypothesize that increased resistance of *lox5-3* mutant may be at least in part due to overproduction of this protein.

Two thioredoxin type-m proteins (TRM1, TRM2) (GRMZM2G181258, GRMZM2G358009) have been found to be accumulated differently in the three genotypes (Fig. 11). TRM1 accumulates in *lox4-7* to levels 2-fold greater compared to B73 or *lox5-3*, but TRM2 accumulated to 77 times greater levels in the *lox5-3* mutants in comparison to either B73 or *lox4-7*. Heterologous expression of yeast thioredoxin type-m in *Arabidopsis* suggested that thioredoxin mediates plant tolerance to oxidative stress induced by either hydrogen peroxide (H_2O_2) or an alkyl

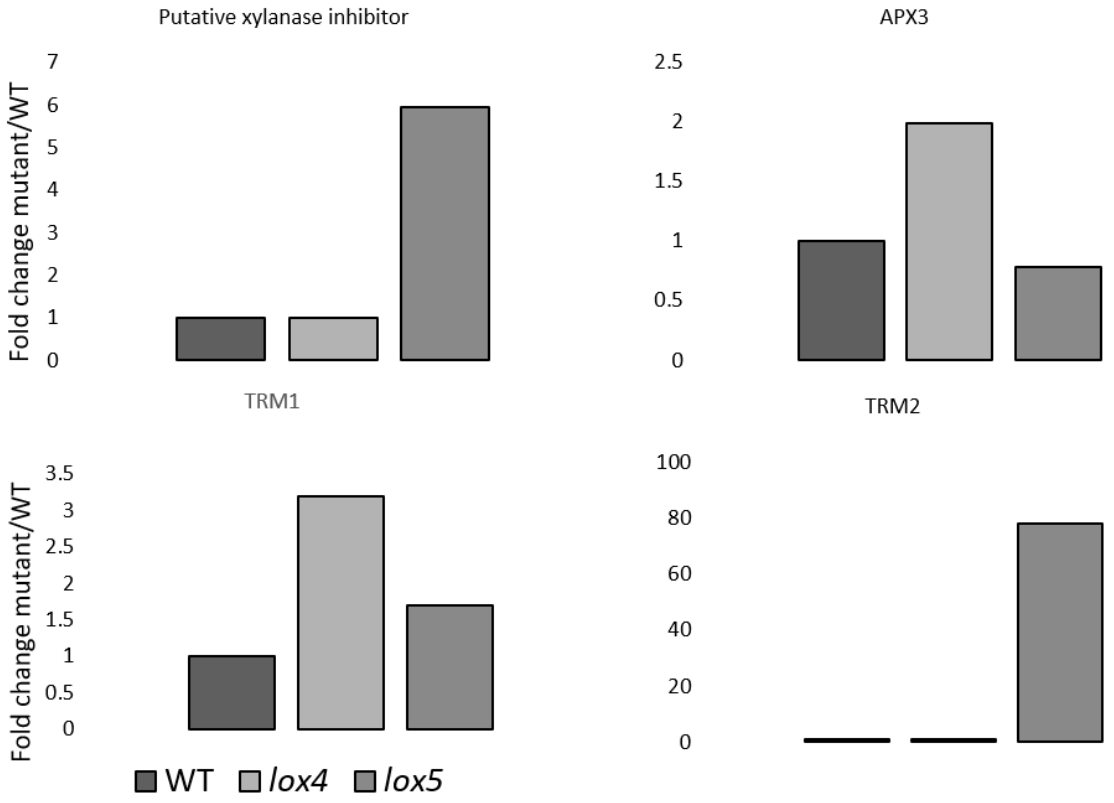

Fig. 11: Fold change of proteins found differentially expressed through shot-gun whole proteome analysis by B73 WT, *lox4-7*, and *lox5-3* mutants. Plants were grown to V4 stage and drop inoculated with 10^6 conidia/ml of *C. graminicola* spore suspension on the third leaf. The leaf was then excised and processed 2 days after inoculation. These proteins are those with possible putative roles in the pathogenesis of *C. graminicola*.

hydroperoxide (Issakidis-Bourguet et al., 2001). An ascorbate peroxidase homolog (APX3) (GRMZM2G137839), another enzyme that assists in ROS scavenging, was also found to differentially accumulate in *lox4-7* mutants compared to both WT and *lox5-3*.

Taken together, many transcripts and proteins found to be differentially expressed or accumulated in *lox4-7* and *lox5*-3 in the RNAseq experiment and the shot-gun whole proteomics experiment are involved in ROS scavenging and oxidative stress. I next investigated whether defense conferred by *ZmLOX4* and the susceptibility conferred by *ZmLOX5* is mediated by ROS.

3.10 Loss-of-function mutants of *lox5* produce more hydrogen peroxide in response to *C. graminicola*

One common thread found in this study is that disruption of *ZmLOX4* and *ZmLOX5* resulted in altered expression of genes involved in ROS production or scavenging. *CAT3* was

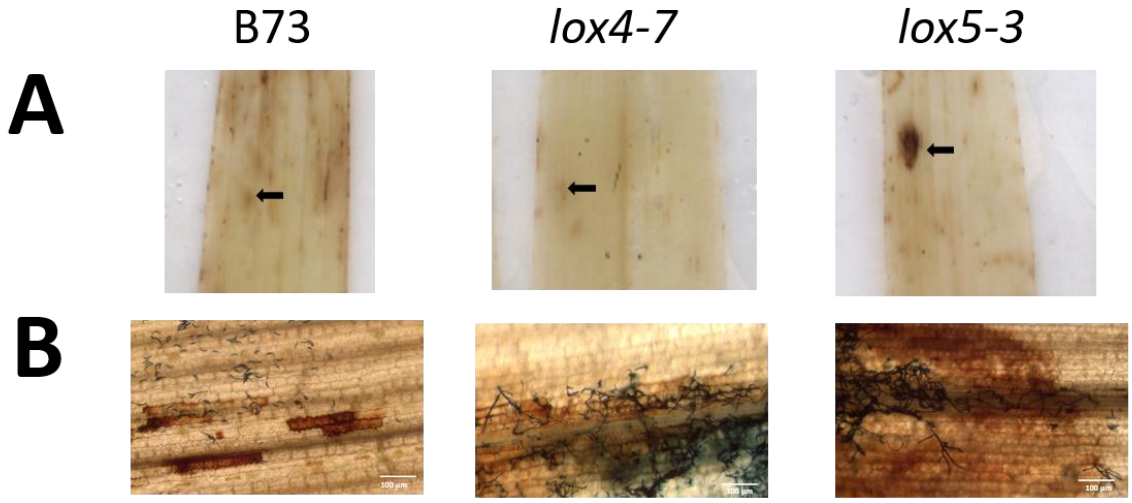

Fig. 12: H_2O_2 accumulates at higher levels in *lox4-7* and *lox5-3* mutants than on WT. (A) DAB-stained leaves of WT, *lox4-7* mutant, and *lox5-3* mutant. Black arrows indicate site of inoculation. (B) 100x magnification of the site incululated by *C. graminicola* spores indicated by black arrows on 11A. Fungal mycelia was stained using lactophenol trypan blue.

found to be differentially expressed in the RNAseq experiment (Fig. 8A) and TRM1, TRM2, and APX3 were found to be differentially expressed in the proteome analysis (Fig. 11). SA was also found to be overproduced in *lox5-3* mutants (Fig. 6) in response to *C. graminicola* infection. One of the functions of SA is to control of H_2O_2 production by binding to catalase enzymes such as CAT3 and inhibiting its ROS-scavenging activity (Chen et al., 1993). *CAT3*, a candidate gene that was expressed differentially in the RNAseq experiment (Fig. 8A), is a protein that scavenges H_2O_2 and thus prevents cell damage resulting from uncontrolled H_2O_2 accumulation. Given that *lox5-3* mutants overproduce SA in response to *C. graminicola* (Fig. 6), it was also tested whether disruptions of *ZmLOX4* and *ZmLOX5* affect H_2O_2 production in response to *C. graminicola*. 3'3-diaminobenzidine (DAB) was used to visualize H_2O_2 accumulating in leaves in response to *C. graminicola* infection at 2 days after inoculation.

A large induction of H_2O_2 is observed in *lox5-3* mutant plant leaves compared to either WT or *lox4-7* mutants in response to *C graminicola* inoculation (Fig. 12A). These inoculation spots were also observed under 100x magnification with light microscopy and by staining fungal hyphae with lactophenol trypan blue. H_2O_2 was observed to spread further beyond the ectopic growth of the fungus on *lox5-3* mutant plants compared to either B73 or *lox4-7* and stronger in *lox5-3* mutants compared to in both B73 and *lox4-7* mutants, as indicated by the darker color stain (Fig. 12B).

3.11 *C. graminicola* appressoria develop at different rates on infected B73, *lox4*, and *lox5* mutants

Appressoria is the fungal structure used by *C. graminicola* to directly penetrate plant cell wall by turgor pressure and its development could be affected by signals the fungus receives from the plant. Since disruption of *ZmLOX4* and *ZmLOX5* were able to affect the accumulation of

defense hormones such as SA basally and in response to *C. graminicola* (Fig. 6), I hypothesized that *C. graminicola* infection processes would differ in maize plants that are disrupted in *ZmLOX4* and *ZmLOX5* functions. In this experiment, WT, *lox4-7*, and *lox5-3* mutant plants were drop-inoculated with conidia suspension of *C. graminicola*, and the individual inoculation spots were excised, depigmented, and observed through light microscopy in order to quantify appressoria that developed at 72 and 96 hours post inoculation.

Significantly more appressoria were found on B73 plants at 72 hpi to *lox5-3* mutant plants and though not statistically significant, numerically *lox4-7* also shows a lower number of appressoria (Fig. 13), suggesting that appressoria development differs between the three

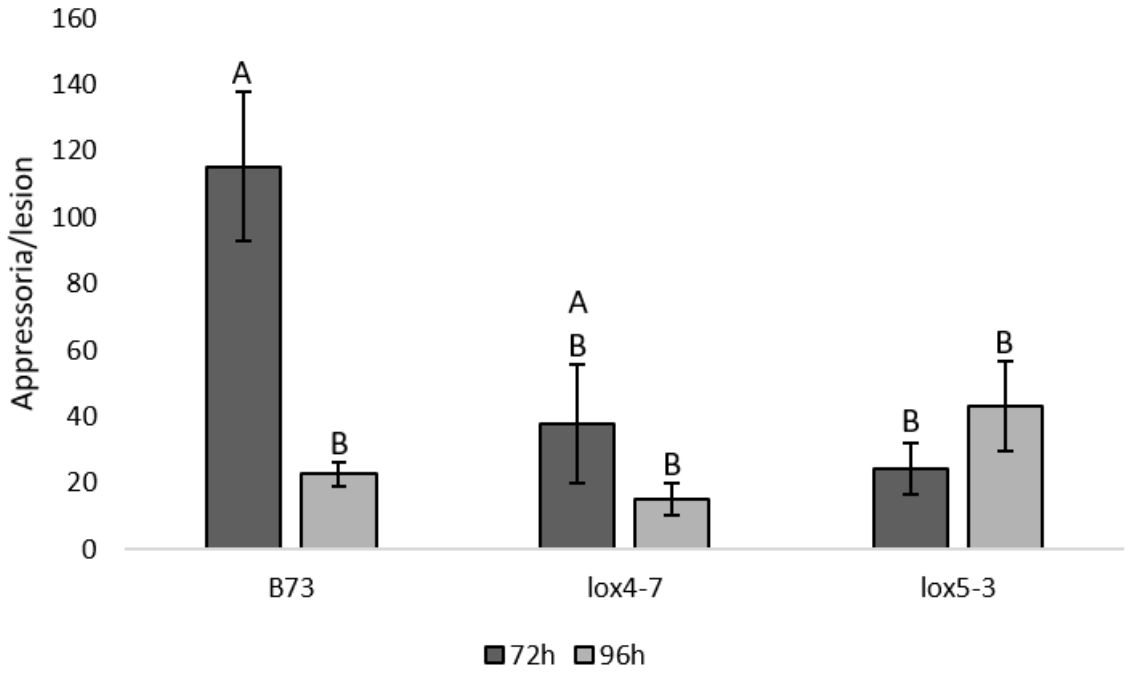

Fig. 13: Appresoria develop at different rates between B73 WT, *lox4-7*, and *lox5-3* mutants. Plants were drop inoculated with 10^5 conidia/ml spore suspension of *C. graminicola*. Appressoria structures were counted for each lesion at 72 and 96 hours post inoculation (hpi). Error bars represent ± standard error from at least three biological replicates (*P<0.05 Tukey's HSD).

genotypes. Interestingly, appressoria numbers drop significantly at 96 hpi on WT, suggesting that either appressoria disappears after 72 hpi or are more easily washed away through the depigmentation process at 96 hpi. If the appressoria is more easily removed, it might indicate that it is no longer useful for *C. graminicola* to complete the infection processes.

4. DISCUSSION AND CONCLUSIONS

Even though ZmLOX4 and ZmLOX5 are close LOX paralogs that share around 95% amino acid identity to one another, they have very different roles in defense against *C. graminicola*. Both previous research by Park (2011) and this study (Figs. 3, 4) showed that *lox4* mutants are more susceptible to *C. graminicola* both in leaves and stalks, while *lox5* mutants are more resistant. Such a difference in their function during the interactions with *C. graminicola* is likely due to their difference in promoter regions (Table 1). Park et al. (2010) showed that the two genes have vastly different expression patterns in diverse maize organs and in response to different stimuli and hormonal treatments. Their expression patterns are also different in response to *C. graminicola*, as shown in Fig. 1. In this study the expression of *ZmLOX4* in plants treated with *C. graminicola* was shown to be induced at early time points (Fig. 1A). Interestingly, *ZmLOX4* is induced rapidly 3h after inoculation (Fig. 1A). The significance of this early induction is not immediately evident because the fungus does not germinate and form appressoria to penetrate the cell wall until around eight hours after inoculation. This suggests that *ZmLOX4* was induced by an early recognition of pathogen infection processes, which I hypothesized to be mediated by PAMP signals such as chitin. In support of this hypothesis, I have found that *ZmLOX4* is induced transiently by chitin oligosaccharides as early as 30 minutes after inoculation (Fig. 2A) and thus, is an early PAMP-responsive gene.

In contrast to *ZmLOX4*, *ZmLOX5* is not induced by *C. graminicola* and is instead repressed in response to *C. graminicola* (Fig. 1B). In addition, *ZmLOX5* is not responsive to chitin (Fig. 2B), but is induced by touch stimuli associated with spray treatment with water, confirming the expression pattern of *ZmLOX5* revealed by previous research (Park, 2011). Taken together, these

results pointed to vastly different functions of these two related genes. Indeed, Park (2011) had shown that *ZmLOX5* is essential for maize defense against chewing insects such as Fall Armyworm, while *ZmLOX4* is not required for defense against insects. *ZmLOX5* early response to touch might be a way for the plant to recognize the presence of an insect herbivore.

It has been shown by Poncini et al. (2017) in Arabidopsis that AtPEP1, a damage-associated molecular pattern (DAMP), is able to more elicit a stronger immune response to either chitin or the bacterial PAMP flg22. It might also be interesting to use this protein, or even the maize homolog of this protein, to see how DAMPs induce *ZmLOX4*, *ZmLOX5*, or various defense responses in the future as well.

I have confirmed that *lox4-7* and *lox5*-3 mutant maize plants at the BC7 genetic stage displayed disease symptoms similar to those previously observed for the BC4 stage plants (Park, 2011) (Figs. 3, 4). Specifically, *lox4-7* mutants are more susceptible to ALB and ASR and, conversely, *lox5-3* mutants are more resistant (Figs. 3, 4). This reaffirms the conclusion that *ZmLOX4* is essential for defense against *C. graminicola* and *ZmLOX5* facilitates pathogenicity.

The main goal of this study was to investigate the downstream 9-oxylipin products of the ZmLOX4- and ZmLOX5-mediated pathways. I sought to find 9-oxylipins which were missing or produced at significantly lower levels in either the *lox4-7* or *lox5-3* mutants compared to WT, indicating that they are the direct, specific products of these two LOX enzyme isoforms. Unfortunately, among the over 60 oxylipins quantified in this study, none of the individual 9-oxylipins were significantly downregulated in *lox4-7* or *lox5-3* mutants basally or in response to *C. graminicola*. The most likely reason of our inability to identify specific products of these two genes is that the oxylipin that is produced by the pathways of ZmLOX4 and ZmLOX5 is not among the ones we were able to quantify. Our laboratory is able to detect and quantify only 60 out of total

over 600 known plant oxylipins due to lack of commercially available standards. Another potential pool of oxylipins we miss in current analytical capabilities is oxylipins esterified to complex membrane lipids. A localization study of ZmLOX4 using GFP on protoplasts by Tolley et al. (2018) reveals that ZmLOX4 localize to the tonoplast. Thus, it is possible that these enzymes act on membrane-bound rather than free fatty acids. Alternatively, it is possible that other 9-LOX genes in maize may compensate for the lack of a functional *ZmLOX4* or *ZmLOX5*. Previous research by Park (2011) has revealed that other 9-LOX genes are overexpressed in the *lox5* mutants, resulting in only a small reduction of wound-induced 9-oxylipins in the *lox5* mutant, which explains why total 9-oxylipin products are not greatly reduced by the disruption of these two isoforms. The other maize 9-LOXs, *ZmLOX3, ZmLOX12*, and the dual-specificity maize LOXs that are able to act as both 9- and 13-LOXs, *ZmLOX1* and *ZmLOX2*, might increase their activity in order to compensate for the loss of *ZmLOX4* and *ZmLOX5*, as has been seen in the work of Park (2011). To disentangle these complex interactions between diverse 9-LOXs, creation of double, triple or even quadruple mutants will be necessary in the future studies.

Despite the shortcomings of the analyses of individual oxylipins, analysis on the total 9-oxylipins products of the different 9-LOX branch pathways illustrated in Fig. 5 reveals that *lox4-7* and *lox5-3* mutants have significantly higher levels of 9-AOS products compared to WT plants, which suggest that the oxylipin products of *ZmLOX4* and *ZmLOX5* both are involved in suppressing 9-AOS activity at 24hpi (Fig. 5A). However, the resistant *lox5-3* mutants produce lower amounts of 9-AOS oxylipins at 48hpi (Fig. 5A). Research by Mims and Vaillancourt (2002) reveals that *C. graminicola* begins to switch its life cycle from biotrophy to necrotrophy around 48 hpi. This might suggest that the production of these 9-AOS oxylipins are not important for

maize defense against *C. graminicola*, and might even be detrimental to the plant in the pathogens' necrotrophic phase.

Other findings from this experiment include the lower accumulation of oxylipin products in both *lox4-7* and *lox5-3* mutants compared to WT in the 9-EAS and the 9-PXG/reductase pathways at 48 hpi (Fig. 5B). Both *ZmLOX4* and *ZmLOX5* may be involved or are able to regulate oxylipins produced in these two branches of the LOX pathway during the pathogen's switch from biotrophy to necrotrophy, suggesting that oxylipins produced in these pathway branches have as yet unknown role in the plant-pathogen interactions.

Though we have not found the specific oxylipin products of *ZmLOX4* or *ZmLOX5*, this study has shed more light on potential molecular and biochemical roles these two LOX isoforms play in the defense against *C. graminicola*. Hormone profiling revealed that accumulation of SA and JA-Ile was substantially altered in *lox4-7* and *lox5-3* mutants and are the most likely mechanisms by which these two genes have such contrasting roles in defense. SA accumulation was increased in *lox5-3* mutants compared to WT in response to *C. graminicola* (Fig. 6A). In contrast, SA levels were much lower in *lox4-7* mutants in all time points (Fig. 6A). SA is a hormone that is known to be important in plant defense against biotrophic and hemibiotrophic pathogens (Glazebrook, 2005) such as *C. graminicola*. Overall, these results indicate that the direct oxylipin product of *ZmLOX4* and *ZmLOX5* have a role in regulating SA production. To the best of my knowledge, the role of a 9-oxylipin in the regulation of SA has not been reported before.

Given that SA is accumulated at higher levels in the resistant *lox5-3* mutant compared to in WT, the next step is to reveal whether exogenous treatment of SA onto plant leaves would be able to induce resistance phenotype in WT and *lox4-7* mutants to levels similar to *lox5-3* mutant. SA treatment to WT enabled the plant to increase its resistance to *C. graminicola* to levels

comparable to *lox5-3* mutants (Fig. 7), showing that SA is important for resistance to *C. graminicola*. SA treatment to *lox4-7* mutants also partially rescued its susceptible phenotype, but not to the level of *lox5-3* mutants, or even to WT levels (Fig. 7). This suggests that the potential mechanism that makes *lox4* mutants more susceptible to *C. graminicola* is not entirely SA-related. Interestingly, SA treatment was not able to induce increased resistance in *lox5-3* mutants (Fig. 7). This suggests that the SA that was already highly accumulated in *lox5-3* mutants is sufficient for defense against *C. graminicola*, and that increased SA does not induce greater resistance.

JA-Ile is a 13-LOX product of the 13-AOS pathway. It is the bioactive form of JA (reviewed in Borrego and Kolomiets, 2016). Given the well-known antagonistic interplay between SA and JA, it is surprising to see an upregulation of both JA-Ile and SA in the *lox5-3* mutant plants. It is possible that disruptions of *ZmLOX5* also disrupt the well-known antagonistic interplay between SA and JA, suggesting that *ZmLOX5* might be an essential gene regulating this antagonistic relationship. Whether JA-Ile has a role in increased resistance of *lox5-3* against *C. graminicola* diseases will require creation of a triple mutant with the *opr7/opr8* double mutant, which has been shown to be deficient in JA (Yan et al., 2012).

Previous research has also shown that *ZmLOX5* is inducible by both SA and JA (Park et al., 2010), the two defense metabolites that were accumulated at high levels in *lox5-3* mutants compared to WT (Fig. 6). The increased accumulation of SA in *lox5-3* mutants suggests that the *ZmLOX5*-derived oxylipin(s) suppresses the accumulation of SA. Supporting this, cis-acting elements have been found upstream of the *ZmLOX5* open reading frame (Table 1) that potentially mediate induction the gene's expression in response to both SA and JA. The high accumulation of SA and JA-Ile in *lox5-3* mutants and its induction by these two molecules suggest that *ZmLOX5* is involved in negative feedback of SA and JA, suppressing the further accumulation of this

hormone in response to their production. When *ZmLOX5* is disrupted, SA and JA-Ile accumulates at a higher level without the *ZmLOX5*-regulated negative feedback. It is possible that *ZmLOX5* is involved in the antagonistic interplay of JA and SA.

To reveal genetic and molecular pathways that are affected by the as-of-yet unknown oxylipins directly produced in the *ZmLOX4* and *ZmLOX5* pathways, transcriptomic and proteomic profiling of B73, *lox4-7*, and *lox5-3* plants were performed with RNA-seq and shot-gun proteomics, respectively. The RNA-seq experiment revealed the overexpression of a putative *ICS1* homolog gene in *lox5-3* mutants (Fig. 8A). ICS is an enzyme that synthesizes isochorismate, a precursor of SA (Wildermuth et al., 2001) and SA synthesized in the ICS pathway has been shown to be responsible for 90% of pathogen-induced SA (Chen et al., 2009). SA is overproduced in *lox5-3* mutants (Fig. 6A) and treatment of SA is able to increase the resistance of WT to *C. graminicola* infection (Fig. 7). It is possible that the increased SA produced in *lox5-3* mutants are due to the upregulation of this ICS homolog.

lox5-3 mutants overexpress *CAT3*, a ROS scavenger and inhibitor of H_2O_2 activity (Fig. 8A). Given greater levels of H_2O_2 in infected *lox5-3* mutants (Fig. 11), it is not clear why *CAT3* is overexpressed in *lox5-3*. Research done by Chen et al., (1993) has revealed that SA can bind to catalase enzymes to inhibit its ROS scavenging activity. It is possible that *CAT3* was produced in response to high H_2O_2 production, and the upregulated SA production in *lox5-3* mutants (Fig. 7) suppressed CAT3 activity, thus allowing increased H_2O_2 production in the mutant.

Genes involved in the benzoxazinoid pathway (*BX10*, *BX11*, *GLU1*) were found to be differentially expressed in WT, *lox4-7*, and *lox5-3* mutants in response to *C. graminicola* inoculation (Fig. 8A). BX10 and BX11 metabolize DIMBOA-Glc to HDMBOA-Glc (Meihls et al., 2013; Handrick et al., 2016). These two BX isoforms have very different expression patterns

in the different genotypes. *BX10* was downregulated in *lox5-3* mutants compared to levels in WT, with *BX11* being upregulated in *lox5-3* mutants. *GLU1*, which synthesizes DIMBOA from DIMBOA-Glc (Meihls et al., 2013) was also found to be downregulated in *lox5-3* mutants compared to in B73 wild type and upregulated in *lox4-7* mutants compared to WT (Fig. 8A). The overexpression of *BX11* in *lox5-3* mutants is not as significant as the downregulation of *BX10* and *GLU1* transcripts. This suggests that *lox5* mutants have more DIMBOA-Glc since it is not being synthesized into DIMBOA or HDMBOA-Glc. Research has shown that DIMBOA-Glc is important in callose production, and its transformation into HDMBOA-Glc does not allow the plant to produce callose at similar levels (Meihls et al., 2013), and its transformation into DIMBOA may be important for callose deposition, as apoplast infiltration of DIMBOA in maize has mimicked chitosan-induced callose deposition (Ahmad at el., 2011). Meanwhile, it has been shown that a fungus that is closely related to *C. graminicola, Magnaporthe oryzae,* utilize plasmodesmata for cell-to-cell movement (Kankanala et al., 2007). While callose deposition in plasmodesmata could be a factor in defense against *M. oryzae,* this data suggests that it is not relevant to the defense against the closely-related *C. graminicola*. It is possible that the resistance of *lox5-3* is regulated by the benzoxazinoid pathway, though not through callose deposition. The callose synthesis pathway has also been shown to be involved in the negative regulation of SA (Nishimura et al., 2003) but conversely, SA has also been reported to be involved in positive regulation of callose deposition in plasmodesmata (Wang et al., 2013). Therefore, I hypothesize that increase of SA accumulation in *lox5-3* may be important in fungal defense through the regulation of the benzoxazinoid pathway. The expression of BX10 was confirmed through qPCR analysis to be highly expressed basally in *lox4-7* mutants (Fig. 9).

One striking group of genes that was found to be differentially expressed in *lox4-7* and *lox5-3* mutants contain a putative tyrosine kinase functional domain (Fig. 8B). In animals, tyrosine kinases are enzymes that transfer a phosphate group from ATP onto a protein, and are usually associated with a tyrosine phosphatase which removes the phosphate group from the protein. So far, no genetic evidence of tyrosine kinases can be found in plants (Shankar et al., 2015), unlike tyrosine phosphatases. The functional domains of the genes discussed here are predicted computationally and may not be true protein tyrosine kinases. Experiments with tyrosine kinase inhibitors in Arabidopsis revealed strong effects on ABA-related processes in plants, including stomata closing (Ghelis et al., 2008). Our results show that a number of genes with this functional domain have opposite expression patterns in *lox4-7* and *lox5-3* mutants compared to WT (i.e. overexpressed in one and underexpressed in the other) (Fig. 8B), suggesting that oxylipins produced in the *ZmLOX4* and *ZmLOX5* pathways have roles in the regulation of protein phosphorylation and dephosphorylation.

Mean difference plots between the expression of genes in WT, *lox4-7*, and *lox5-3* mutant using EdgeR revealed the extent of transcriptional reprogramming that are caused by the disruptions of *ZmLOX4* and *ZmLOX5* (Figs. 9A, B, C). Comparisons between each genotype reveal that over 2000 genes between each pair (WT and *lox4-7*, WT and *lox5-3*, *lox4-7* and *lox5-3*) are differentially regulated and showed that both mutation caused extensive changes in gene expression (Figs. 9A, B). Even with the high degree of identity between ZmLOX4 and ZmLOX5 proteins, the disruption of these two isoforms affect very different genes (Figs. 9C, D, E), revealing that ZmLOX4 and ZmLOX5 play roles in different regulatory networks. This suggests that these genes regulate a complex network of genes with many different functionalities. Analysis on the raw counts of transcripts between the mutant genotypes and WT also reveal that even though both

lox4-7 and *lox5-3* share transcripts in common which their disruption affects, most of the transcripts that they affect are mutually exclusive (Figs. 9D, E). This means that even though ZmLOX4 and ZmLOX5 proteins share around 94% identity (Park, et al., 2010), the specific oxylipin products of these genes affect very different gene networks.

The proteome profile of WT, *lox4-7*, and *lox5-3* mutant plants were analyzed through shot-gun proteomics at 3 days after inoculation with *C. graminicola*. A putative xylanase inhibitor was overexpressed in *lox5-3* mutants compared in WT (Fig. 11). Xylanase is a cell wall degrading enzyme that was shown in another member of the *Colletotrichum* genus, *C. lagenarium* to be a pathogenicity factor (Doux-gayat et al., 1978). A study characterizing the secreted proteins of *C. graminicola* has also provided evidence that *C. graminicola* also secretes a xylanase (Krijger et al., 2008). The increased production of the inhibitor of this enzyme might contribute to the resistance of *lox5-3* against *C. graminicola*. I have also found that proteins which control oxidation and ROS scavenging, such as TRM1, TRM2, and APX3 are differentially accumulated in *lox4-7* and *lox5-3* mutants compared to in WT (Fig. 11). While the thioredoxin here is a type-m, another type of thioredoxin, thioredoxin type-h, has been recently found to be important in maize defense against Sugarcane Mosaic Virus (Liu et al., 2017). Both SA and *CAT3* were differentially regulated in *lox4-7* and *lox5-3* mutants. The H_2O_2-scavenging activity of catalase is inhibited by the binding of SA to this enzyme. Because genes important for ROS production and scavenging were found to be expressed differentially in the shot-gun whole proteome profiling and RNA-seq (such as TRM1, TRM2, APX3) (Figs. 8A, 10), H_2O_2 accumulation was analyzed in response to *C. graminicola* infection. It was found that *lox5-3* plants had greater accumulation of H_2O_2 (Fig. 12A), with H_2O_2 spreading farther beyond the range of fungal growth (Fig. 12B). Considering the hemibiotrophic nature of the pathogen, it is reasonable to suggest that such peculiar H_2O_2

accumulation in the *lox5* mutants may prevent fungal spread beyond infection site, creating a zone that is unusable by the biotrophic edge of the pathogen colony. This together with higher SA levels in the *lox5* mutant may be the major reason behind increased resistance to the pathogen.

Light microscopy images of fungal staining on Fig. 12B revealed that *C. graminicola* develops at different rates on B73 wild type, *lox4-7*, and *lox5-3* mutant plants. At the 48hpi in which the leaves were observed, the conidia *C. graminicola* on B73 plants have not yet germinated, but hyphal growth has already progressed further on both *lox4-7* and *lox5-3* mutants compared to WT (Fig. 12B). This prompted the hypothesis that fungal penetration may also have been accelerated in the two mutants. This hypothesis was supported by significantly lower number of appressoria observed on both mutants as compared to WT (Fig. 13). Given the slow development of *C. graminicola* on WT plants with conidia not germinating and forming appressoria at 48hpi (Fig. 12B), it is possible that development of *C. graminicola* is enhanced in both mutants, though the final outcome, increased resistance or the facilitation of pathogenicity, is opposite in both *lox4-7* and *lox5-3* (Figs. 3, 4). It is possible that ZmLOX4 and ZmLOX5 are both able to change the signals detected by the fungus on the surface of the leaves and affect their growth and development. This means either that ZmLOX4 and ZmLOX5 are able to change the signals secreted by the leaf or change the wax and cuticular composition detected by the pathogen, affecting the fungus' growth and development.

Through these results, I hypothesize that *ZmLOX5*-derived oxylipins inhibit the production JA-Ile and SA, the latter which binds to and inhibits CAT3 activity (Chen et al., 1993) and enables the rapid production of H_2O_2. I hypothesize that these factors contribute to how *the ZmLOX5*-derived oxylipins facilitate the pathogenicity of *C. graminicola*. On the other hand, I hypothesize that *ZmLOX4*-derived oxylipins induce the production of SA, the major defense compound against

biotrophic and hemibiotrophic pathogens such as *C. graminicola*, and though SA does have a role in the resistance against *C. graminicola* that is conferred by *ZmLOX4*, it is not the complete story. To fully investigate the story of how these two genes are involved with the SA with regards to *C. graminicola* disease resistance, double and triple mutants must be made with NahG mutant maize.

Other potential future avenues of research are to focus on how *ZmLOX4* and *ZmLOX5* affect maize callose deposition in response to *C. graminicola* and how *ZmLOX4* and *ZmLOX5* affect leaf wax, cuticles, or other leaf surface signals to affect the development of *C. graminicola*.

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